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Metabolomics in the study of retinal health and disease

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ABSTRACT

Metabolomics is the qualitative and quantitative assessment of the metabolites (small molecules < 1.5 kDa) in body fluids. The metabolites are the downstream of the genetic transcription and translation processes and also downstream of the interactions with environmental exposures; thus, they are thought to closely relate to the phenotype, especially for multifactorial diseases. In the last decade, metabolomics has been increasingly used to identify biomarkers in disease, and it is currently recognized as a very powerful tool with great potential for clinical translation. The metabolome and the associated pathways also help improve our understanding of the pathophysiology and mechanisms of disease.

While there has been increasing interest and research in metabolomics of the eye, the application of metabolomics to retinal diseases has been limited, even though these are leading causes of blindness. In this manuscript, we perform a comprehensive summary of the tools and knowledge required to perform a metabolomics study, and we highlight essential statistical methods for rigorous study design and data analysis. We review available protocols, summarize the best approaches, and address the current unmet need for information on collection and processing of tissues and biofluids that can be used for metabolomics of retinal diseases. Additionally, we critically analyze recent work in this field, both in animal models and in human clinical disease, including diabetic retinopathy and age-related macular degeneration. Finally, we identify opportunities for future research applying metabolomics to improve our current assessment and understanding of mechanisms of vitreoretinal diseases, and to hence improve patient assessment and care.

1. Metabolomics: concepts and opportunities

Human biology is diverse and complex. Most conditions and diseases have a multifactorial etiology, driven by a combination of genetic and environmental factors, which interact to lead to a range of phenotypes (Dunn et al., 2011a). Indeed, it is well-recognized that environmental exposures can induce epigenetic modifications, and influence the transcription of DNA into RNA, and its translation into proteins (Crick, 1970; Jafari et al., 2017). Downstream of all the genetic transcription and translation processes are the metabolites, low molecular weight molecules (< 1–1.5 kDa), which thereby reflect both the highly dynamic and interactive system of biological molecular layers (i.e. genome, transcription, translation, and metabolism), and the influence of external factors in these processes (i.e. environment, diet, age and microbiome, among others) (Patti et al., 2012) – Fig. 1.

The multidisciplinary field studying the metabolome is known as "metabolomics" (nowadays used interchangeably with metabonomics), and can be defined as the study of the global qualitative and quantitative composition of metabolites in a biological system (Fiehn, 2002). The total number of metabolites varies among different biological specimens, and remains unknown. According to the Human Metabolome Database (http://www.hmdb.ca/statistics#metabolite-statistics; assessed on 11/11/2018), one of the largest metabolite databases worldwide, humans have at least 114,100 metabolites (confirmed or

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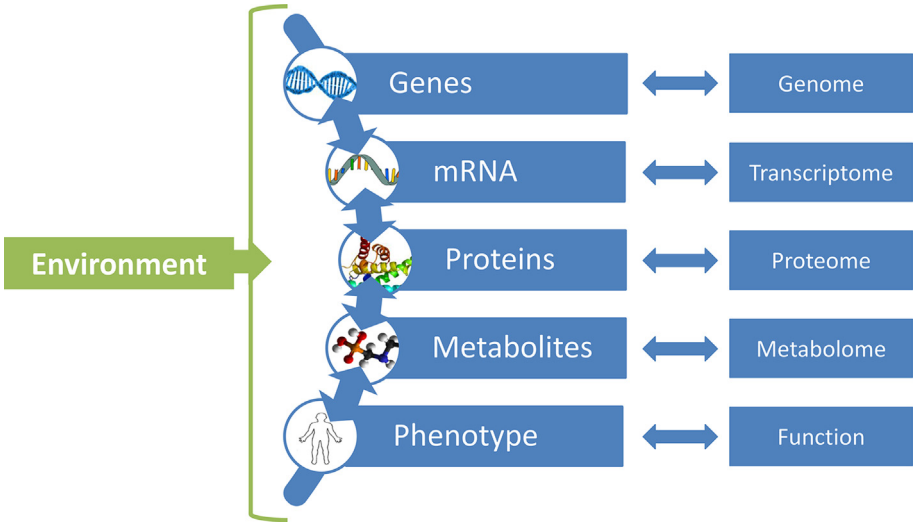

Fig. 1. Schematic representation of 'omics in system biology. As shown, metabolomics is the downstream of the genetic transcription process and its interactions with environmental exposures.

expected). However, this number includes exogenous metabolites (such as toxins and drugs), and only 92,372 are thought to be endogenous. The latter include breakdown products of nucleotides, amino acids, carbohydrates, and lipids. Due to their biological relevance, the study of lipid metabolites has its own subfield of study, known as "lipidomics" (Hu and Zhang, 2018). Importantly, each body compartment has its own metabolome, but they are connected through the vascular and lymphatic systems, which also have a specific metabolomic profile (Nicholson et al., 2012a). In contrast to the genotype, which remains relatively stable over a lifespan, the metabotype (metabolomic phenotype) varies with time, and every metabolomic characterization of a biosample represents a snapshot of that particular state and time (Suhre and Gieger, 2012).

Compared to genomics and proteomics, metabolomics is a relatively new field (Kell and Oliver, 2016), but it is becoming an increasingly important tool in medicine. Among the 'omics, the metabolome is perhaps the most closely linked to the phenotype and thus, can provide information on normal and pathological conditions, as well as on the effect and response to external stimuli (Trivedi et al., 2017). Indeed, the study of the variations of the metabolome has major advantages, including the possibility of: (i) increasing the understanding of the pathophysiology of a disease at a molecular level and generating new hypotheses for disease mechanisms; (ii) identifying biomarkers of disease risk prediction and diagnosis; (iii) assessing disease progression; (iv) interpreting the influence of environment and lifestyle exposures in disease; (v) assessing drug efficacy, toxicity and adverse drug reactions (Nicholson et al., 2012a; Jové et al., 2014; Kohler et al., 2016). Metabolomic profiles can be measured from easily accessible biofluids or tissues that can be sampled readily in the outpatient setting, which represents an additional advantage (Nicholson et al., 2012a). The strength of association with disease outcomes also tends to be higher in metabolomics than in genetics. Thus, metabolomic studies require smaller sample sizes than genetic studies (Manolio et al., 2009).

Metabolomics has an important role to play in personalized and stratified medicine (Ziegelstein, 2017). Clinical diagnosis, prognosis prediction, and treatment selection are improved by tools that can help classify diseases and their subtypes, as well as define underlying individual variations in patient biology and responses. Metabolomics is one of these tools (Nicholson et al., 2012a), and also aids our understanding by providing insight into the interactions between genetic, environmental and lifestyle factors (Suhre and Gieger, 2012). Because of this, metabolomics has been employed in several medical fields (Trivedi et al., 2017), including in large epidemiological and population-based studies (Patel et al., 2017; Suhre et al., 2010). Metabolites, as the final products and "effectors" of metabolism, represent a "unique currency" in distinguishing the pathological from the normal state. While genomics can provide guidance as to where to look for disease-associated variants, it is the metabolic profile of a tissue under normal and abnormal conditions that is the final common denominator distinguishing health from diseased states and, thus, can provide insight into mechanism of disease and potential therapeutic targets for intervention. Cataloguing metabolic profiles in different normal and abnormal states will enable us to validate and interpret genomic variants and place them in a physiologically relevant context (Holmes et al., 2016).

The utility of metabolomics in ophthalmology has also been explored and reviewed (Tan et al., 2016a,b; Midelfart, 2009; Young and Wallace, 2009). However, despite the relevance of these manuscripts, a comprehensive summary of the methodological approaches required to perform high-quality metabolomics studies applied to the field of vitreoretinal diseases was missing in the literature. Namely, procedures for collection of biospecimens of interest have not been fully addressed, and prior reviews did not discuss essential aspects of data analysis' methodologies, and opportunities for data biological interpretation. Additionally, in the last two years, novel work has been published, and new approaches have been developed. The potential of metabolomics for the development and application of precision medicine to vitreoretinal diseases also needs to be further explored.

In this manuscript, we set out to compile a comprehensive and detailed summary of the tools and knowledge required to perform a metabolomics study, including those specific to retinal diseases. We have also critically analyzed published work to date, and discuss how a better comprehension of metabolomics can help in our understanding of eye diseases. We hope that this manuscript informs future studies, and contributes to a better understanding of how metabolomics can be useful for ophthalmology in general, and the field of vitreoretinal diseases in particular, as well as how it can fit into clinical practice and hence improve patient assessment and care.

2. Analytical tools for metabolomic profiling

The study of metabolites has a long history, but in the past was limited to the assessment of specific compounds or biochemical pathways known *a priori*, and was therefore targeted metabolomics (Wilcken et al., 2003). A good example is the study of blood glucose levels for the diagnosis of diabetes, or lipoproteins' levels for the

assessment of dyslipidemia. These targeted assessments are well-validated, thus representing an additional advantage for modern, mostly untargeted metabolomics, as they can pave the way for its clinical translation. With untargeted or global approaches, studies measure as many metabolites as possible, and compare them among samples without bias. Such untargeted analysis is predicated on the development of "metabolite libraries" that contain well-characterized metabolite profiles. These may be used as standards, against which profiles of specimen metabolites may be compared for identification. Untargeted metabolomics studies have led to new discoveries, linking cellular pathways to biological mechanisms, and shaping our understanding of physiology and medicine (Patti et al., 2012).

Two main analytical tools are available for metabolomic profiling: nuclear magnetic resonance (NMR) spectroscopy and mass spectrometry (MS) (Emwas, 2015; Barnes et al., 2016a). Depending on the instrument or protocol used, typically one is able to identify from 50 to up to 5000 different metabolites at a time, but no tool, metric or platform is able to identify all existing metabolites in a single analysis run or using a single technology platform. The majority of metabolomic studies use a single analytical source. However, there is a growing acknowledgment of the value of combining NMR and MS (Marshall and Powers, 2017). They are complementary techniques, so combining them is likely to improve the overall quality of a study and enhance the coverage of the metabolome (Dunn et al., 2011a).

Regardless of the technical analytical tool chosen, minimum requirements for reporting a metabolite profiling experiment include sample preparation, experimental analysis, quality control, metabolite identification, and data pre-processing (Khamis et al., 2017). Validation of metabolomic profiles should include at least a description of the calibration model (linearity and range), repeatability and intermediate precision, accuracy and lower limit of quantification (Scalbert et al., 2009).

2.1. Nuclear magnetic resonance (NMR) spectroscopy metabolomics

NMR spectroscopy measures the behavior of nuclei of atoms when they are subjected to a magnetic field, i.e. their spin resonance. When submitted to an external magnetic field, atoms with an odd mass, such as hydrogen (1H) or carbon (13C), behave as dipoles and align along the axis of the applied magnetic field (excitation). This higher energy level is less stable, so these atoms then undergo relaxation, generating radiofrequency signals, which can be expressed as a frequency spectrum (Tognarelli et al., 2015; Barnes et al., 2016a). Currently, instruments using frequencies of 500 and 600 MHz are the most widely used because they represent a good compromise between sensitivity and cost. However, it is important to note that the higher the magnetic field strength (the highest-frequency commercially available instrument operates at 1,000 MHz), the greater the resolution (Barnes et al., 2016a).

Hydrogen is the most abundant atom in living organisms, so proton NMR is the most commonly used NMR technique (Barnes et al., 2016a). In a 1H spectrum, the position of each peak (chemical shift) represents the hydrogen atoms' environment (i.e., proximity of electronegative groups such as nitrogen, oxygen, double bonds, etc.). The size of the peaks, most precisely the area under the curve, provides important information about the number of hydrogen atoms in each environment.

NMR spectroscopy can be applied to liquid samples, but also to solid, gas phase and tissue samples (Emwas, 2015). One of its biggest advantages is the minimal sample preparation required, and the preservation of samples (i.e., even after analysis, samples can still be used for other studies). NMR is also recognized to be highly reproducible and less susceptible to instrument variability. Additionally, it has the advantage of being quantitative, measuring the amount of protons under given conditions, and thus enabling direct spectral data comparisons (Holmes et al., 2016). NMR's major disadvantage relates to its sensitivity, which is lower than that of MS. The number of metabolites visible in the proton NMR spectrum ranges from about 50 in serum/plasma samples to roughly 200 in urine (Kohler et al., 2016). Also, the interpretation of NMR spectra is considered complex and requires substantial training, as signals from different metabolites can overlap (Markley et al., 2017).

2.2. Mass spectrometry (MS) metabolomics

MS identifies metabolites primarily based on their mass to charge (m/z) ratio (Crutchfield et al., 2016). A good analogy is to imagine a cannonball and a tennis ball travelling together, which we hope to deflect with a jet of water. The cannonball is so heavy that it will hardly be deflected at all from its original course. Conversely, the tennis ball is light, and will have a large deflection. In this example, the mass of each ball determines the ability to deflect them. The same principle applies to MS. Atoms and molecules can be deflected by magnetic fields. First, they need to be turned into ions (ionization), and then these are accelerated so that they all have the same kinetic energy. The ions are then deflected by a magnetic field according to their masses and their number of positive charges (Gika et al., 2014; Lind et al., 2016; Wang et al., 2011b). Several techniques are available for ionization (Kohler et al., 2016), with electrospray ionization (ESI) being one of the most commonly used (Wilm, 2011).

A MS spectrum of a sample can be obtained by direct injection, but it is usually performed in tandem with separation techniques, such as liquid chromatography (LC), gas chromatography (GC) or capillary electrophoresis (CE). Importantly, no separation method (GC, LC or CE) enables the simultaneous separation of all metabolites. In addition, there is no one mass analyzer that can measure all metabolites, as some metabolites may not ionize with certain methods, or because their concentration is too low (Khamis et al., 2017).

In biosciences, LC, particularly ultra-high-performance liquid chromatography (UPLC), is becoming increasingly popular and is probably the most widely used method coupled with MS technology (Patti et al., 2012). This is primarily due to the ability of LC to separate and detect a wide range of metabolites, (Jové et al., 2014; Nicholson et al., 2012a) and because of the large number of accessible instruments and open-source data processing software available for this technique (Jové et al., 2014). A disadvantage of LC–MS is ion suppression, as ionization of metabolites may depend on the presence of matrix compounds, particularly with ESI (Scalbert et al., 2009). GC has high separation efficiency and reproducible retention times (Khamis et al., 2017). However, it presents three main pitfalls: possible loss of thermolabile analytes; complex sample preparation; and higher variability compared with LC-based metabolomics (Dunn et al., 2011b). Also of note, concerns have emerged about degradation of metabolites during GC–MS analysis, due to the required exposure to elevated temperatures (Fang et al., 2015). CE has had a long history in the analysis of individual metabolites and can be applied to very low sample volumes. So far, however, its application has been limited, mostly due to the poor sensitivity of the CE-ESI interface. New interfaces have now been developed and are commercially available, thus opening a great future for this method, in particularly if used as a complement to GC and LC-MS (Barnes et al., 2016a).

In general, MS has a much higher sensitivity (detection level of picomoles to femtomoles) than NMR, therefore enabling the measurement of a broader range of metabolites. Additionally, the different MS technologies provide an array of operational principles that can be applied, thus increasing the number of metabolites that can potentially be detected. This is particularly relevant for biological samples(Emwas, 2015). Importantly, MS requires the use of quality control samples, (Barnes et al., 2016a; Gika et al., 2014) as well as multiple simultaneous internal reference standards (typically at least three). Although the ionization required before MS analysis can lead to complications with quantitation, if these reference standards are used along with other validation parameters, targeted MS becomes quantitative. This is

particularly valid for UPLC, which enables a high degree of chemical specificity (Holmes et al., 2016; Kapoore and Vaidyanathan, 2016). Indeed, the superior chromatographic resolution and rapid separation of UPLC, combined with the high sensitivity of MS, allow both the detection and measurement of levels of thousands of metabolites in minutes. Despite this, one needs to be aware that untargeted metabolomics in general is not quantitative, so effect sizes should not be compared across different studies and platforms (Holmes et al., 2016).

2.3. Mass spectrometry imaging metabolomics

Mass spectrometry imaging (MSI) is a method by which molecular information is obtained over two or three spatial dimensions, thus enabling one to determine the distribution of small molecules within a tissue (Petras et al., 2017). For each ion in the collected mass to charge (m/z) range, reconstruction of its intensity in every x-y coordinate pair creates an image of its distribution, thus permitting the virtual dissection of tissues based on macular mass signatures (Sun et al., 2014). This leads to one of the big advantages of MSI, the ability to correlate non-targeted metabolite information with histological data; and it is possible because, unlike traditional MS, sample preparation methods do not result in loss of spatial localization. In a single experiment, it is possible to detect the spatial distribution of thousands of molecules (Naru et al., 2017).

Several MSI techniques have been developed, which mostly differ in their spatial resolution, molecular specificity and sensitivity (Naru et al., 2017). Matrix-assisted laser desorption ionization (MALDI) is currently the most widely applied method, and typically provides a lateral spatial resolution ranging from a few μm to mm. This is dependent on the technique applied, particularly on the laser diameter (Petras et al., 2017). In general, most MALDI studies are performed on linear MALDI time-of-flight (TOF) platforms, which enable a resolution to the level of proteomics. Bowrey et al. (2016) recently reviewed the application of MSI proteomics to the study of the visual system. However, for metabolomics, Fourier-transform ion cyclotron resonance (FTICR) or quadrupole-TOP is recommended, as they both provide higher resolution and mass accuracy (Wang et al., 2011a). MALDI, however, still has important limitations, including instrumentation resolution and requirements for sample preparation (Petras et al., 2017; Zemski Berry et al., 2014). Namely, as the name suggests ("matrix-assisted" laser desorption ionization), this technique requires the application of a matrix to coat the samples to promote biomolecule desorption and ionization prior to analysis (Murphy et al., 2011). This is important because it requires extreme care to ensure that this matrix is applied homogeneously across the tissue, and that no delocalization is introduced; otherwise accuracy and reproducibility are compromised (Gessel et al., 2014). Also, the time required for data acquisition increases following an inverse squared relationship with lateral spatial resolution, and the sensitivity of mass analysis increases with decreased lateral spatial resolution. Additionally, MALDI quantitative analysis is worse than with the traditional extraction and analysis by liquid chromatography MS (Petras et al., 2017).

Recent advances in MSI include developments of techniques other than MALDI, including: secondary ion mass spectrometry (SIMS) and Desorption Electrospray Ionization MS (DESI). SIMS is considered complementary to MALDI, as it offers a higher spatial resolution (less than 1 μm), thereby enabling assessments at cellular levels, and does not require labelling of the compounds to be detected. However, it has a lower chemical specificity (Lockyer, 2014; Kraft and Klitzing, 2014). DESI is considered a very promising technique, and also has the great advantage of not requiring sample preparation and causing minimal tissue architectural disruption. It allows the same tissue section to be first subject to MSI and then to conventional histology, allowing precise structural correlations (Holmes et al., 2016; Jarmusch et al., 2016).

3. Sample collection and processing

3.1. General concepts

Unlike the genetic sequence, metabolomic profiles vary depending on the biofluid being assessed. Appropriate sample collection and processing is an important requirement for successful metabolomic studies (Khamis et al., 2017). All efforts should be made to ensure that the collected samples are qualitatively and quantitatively representative of their source (Ammerlaan et al., 2014), and that bias and uncontrolled experimental variances are minimized (Yu et al., 2011).

Protocols should be developed and reviewed before beginning any collection, as they vary. For example, if metabolomic profiling is going to be performed by a different laboratory/institution than the one that is obtaining the samples, the entire protocol should be discussed with all the involved parties and defined *a priori*. Standard operating procedures (including check lists and labeling procedures) are essential to prevent mistakes and increase efficiency. Supply sources should be uniform and the collection and storage conditions should be well defined. In large-scale human metabolomics studies, samples are frequently collected at different sites. It is therefore important to provide written procedures or guidelines to all researchers, clinicians and laboratory staff involved in the project to ensure the highest reproducibility throughout sample collection and handling (Kohler et al., 2016). Despite the definition of standardized protocols, their strict execution within single- or multi-center clinical studies can be challenging, so monitoring and samples quality assessment is also crucial (Jobard et al., 2016).

For studies using samples already collected as part of biobanks, it is essential to obtain all possible information about the conditions of sample collection and storage (Yu et al., 2011). Since a relatively long time may elapse between the collection of the samples and their analysis, multiple parameters must be monitored to lower the risks for degradation and interconversion, including temperature, light, humidity, time span, quenching and number of freeze–thaw cycles (Kohler et al., 2016). A consensus is still lacking on the effect of freeze–thaw cycles on the metabolome, so multiple freeze–thaw cycles should be avoided as much as possible (Kohler et al., 2016).

In the following section, we discuss the specific requirements to obtain biofluids and other tissue samples that can be relevant for the study of retinal diseases. Metabolomics workbench (www.metabolomicsworkbench.org) is a website supported by the National Institute of Health that has a large and excellent resource of experimental protocols and specific guidelines for sample preparation and metabolites extraction. Of note, metabolomics data can be combined with data from other 'omics methods, such as genomics or proteomics, increasing the ability to investigate the pathophysiology and mechanisms of diseases. To this end, specific methods have been developed, namely the simultaneous metabolite, protein, lipid extraction procedure (SIMPLEX), which enables the assessment of multiple molecular classes from a single sample in parallel (Coman et al., 2016). This is of particular interest to the study of vitreoretinal diseases, where reduced amounts of sample are required, and as mentioned below, eye tissue typically is typically limited in quantity.

3.2. Animal tissue

It is essential to plan animal tissue collection in advance and consider euthanasia method, tissues desired, *post mortem* time to quenching/freezing and consumable selection.

Compounds commonly used for euthanasia are known to have broad effects on cell function and metabolite levels. Isoflurane affects glucose metabolism in mice, and if metabolites of interest are linked to glucose, their metabolism may also be affected in the tissue being studied (Federation of American Societies for Experimental Biology. et al., 2010). Ideally, rapid cervical dislocation or a guillotine should be

used in mice, but these protocols need to be discussed and approved by the Institutional Animal Care and Use Committee. Response to stress also affects metabolism and balancing the euthanasia approach with minimization of stress to the animal is essential for reliable outcomes with animal models (Ghosal et al., 2015; Hurst and West, 2010).

Many metabolites have very rapid turnover rates. Therefore, as with other sample collection, it is key to handle tissue samples as quickly as possible to prevent metabolite degradation. Snap freezing samples in liquid nitrogen is commonly considered best practice. However, if and when that is not possible, cooling to 4 °C maintains most but not all metabolites in the retina for up to 8 hours (Tan et al., 2016a,b). Collecting the entire eye and snap freezing in liquid nitrogen is the quickest and most reliable method and should be used if the broadest metabolite profile possible is desired (Paris et al., 2016). Micro-dissection of specific tissues such as the cornea, lens, vitreous, retina and choroid allows for tissue-specific metabolite analysis but increases *post mortem* processing time and potential changes in metabolite levels. Collection speed must be balanced with sample integrity. If specific tissues are to be dissected, it is best to practice ahead of time to increase speed and accuracy.

The consumables used for tissue collection should be selected based on the subsequent extraction and analytical platforms for analyses. For broad metabolomics analysis including non-polar and lipid species, glass is the best material to work with. Not all glass products are equivalent and it is advisable to consult with those running the extraction and analysis for their recommendations. If the analyses focus on polar metabolites, plastic tubes may be appropriate, but not all plastics are equivalent. The same container type should be used throughout all samples in an experiment and a vessel-alone extraction in parallel can help to distinguish non-tissue derived metabolites.

3.3. Human tissue and samples

3.3.1. Plasma and serum

Blood provides a snapshot of the metabolism that integrates many tissues in the human body, and thus offers a global metabolomic picture (Chetwynd et al., 2017). Therefore, along with urine (also an integrative biofluid), blood is one of the most commonly used biofluids for metabolomics in biomedical sciences (Dunn et al., 2011a). The influence of the pre-analytics practices for blood samples has been discussed in the literature (Hebels et al., 2013), and is extremely important (Kohler et al., 2016).

Either serum or plasma can be obtained from blood, and the preferred fluid for metabolomics remains to be established - plasma seems to be more reproducible, and serum to have higher concentrations of metabolites(Yu et al., 2011). The main difference between them refers to the presence or absence of clotting factors. For serum, whole blood is collected into tubes and is allowed to clot (Chetwynd et al., 2017). One must record the clotting time and temperature at which the clotting occurred, and this should be standardized across all samples (Khamis et al., 2017). Plasma is obtained when whole blood is mixed with an anticoagulant to inhibit clotting. Typical anticoagulants include lithium heparin, EDTA and citrate. In general, the use of lithium heparin is recommended, (Chetwynd et al., 2017; Yu et al., 2011) especially for NMR, even though heparin may lead to ion suppression (Barnes et al., 2016a). Citrate and EDTA have molecular masses similar to some metabolites, and citrate is an endogenous metabolite; therefore, when these anticoagulants are used, caution is required on data analysis. EDTA has the advantage of chelating the potential divalent metal cations present in the sample, which otherwise might accelerate the hydrolysis of important energy metabolites. However, EDTA may also lead to ion suppression (Kohler et al., 2016).

Importantly, blood collection tubes can also release materials into the samples and interfere with metabolomic analysis, so the same tubes should be used across a study and this information should be registered. Circadian oscillations can also significantly influence the metabolome, particularly for lipids, therefore it is important to collect samples at the same time of day and document this information (Kohler et al., 2016). Recent dietary exposures can also affect the results, and if possible, samples should be collected after overnight fasting; otherwise, the time since last meal should be documented. Additionally, for a more complete understanding of the role of nutrition on the profiles obtained, a nutritional assessment should be performed, for example, using a validated food frequency questionnaire (Scalbert et al., 2014).

After collection into the appropriate tubes (without or with anticoagulant), serum or plasma are separated by centrifugation from the blood clot or cell mass, respectively. Samples should be immediately stored as 0.5 or 1.0 mL aliquots at −80 °C or in liquid nitrogen. NMR studies require larger sample volumes (Barnes et al., 2016a). A delay in time between aliquoting and storage can affect the quality of the samples (Yu et al., 2011). Some authors have argued that centrifugation parameters (such as rotation speed and temperature) are not particularly relevant (Jobard et al., 2016), but this remains controversial (Ammerlaan et al., 2014).

3.3.2. Urine

Urinary metabolomic profiling has expanded in the last few years (Slupsky et al., 2007; Khamis et al., 2017). Urine is easily obtained at a reasonable cost (Emwas et al., 2016). Moreover, compared to blood, urine is not subject to many homeostatic mechanisms, and greater varieties of endogenous metabolites can be present, so some authors argue that it may better reflect the changes in human metabolism (Mal, 2016).

Typically three types of urine samples can be collected, and they have important differences among them: first morning void, spot urine and 24-h urine collection (Chetwynd et al., 2017; Slupsky et al., 2007) .The last (24 h collection) is the ideal, as it reduces the impact of any circadian variation and represents a complete circadian cycle. However, it is often not feasible to obtain 24 h samples for clinical studies (Kohler et al., 2016), so first morning void is the choice in most cases. This option can reduce the effect of meals or medication, particularly if collected following an overnight fast. Spot urine samples refer to those taken at any time point, which makes them highly influenced by the variable daily excretion rate, as well as by other environmental factors (Slupsky et al., 2007; Chan et al., 2011; Giskeødegård et al., 2015).

For urine collection, subjects are simply asked to provide a mid-stream urine sample in a sample cup. For immobile subjects it can be collected with a catheter, and for babies urine can be collected with absorbent pads in nappies (Chetwynd et al., 2017). After collection, samples should be frozen at −80 °C as soon as possible, as prior work has shown that urine modifications can occur in a short period of time (Gika et al., 2008), especially at room temperature. At −20 °C, samples can remain stable for a relatively long time, but −80 °C remains the best choice (Gika et al., 2008). Freeze thaw cycles should be kept to a minimum (Khamis et al., 2017).

In a healthy individual, urine is sterile, however it can become contaminated during urination, which can modify the urinary metabolites. In addition to storage at −80 °C and collection of mid-stream samples, which are both beneficial (Kohler et al., 2016), antibacterial additives such as sodium azide and sodium fluoride seem to increase the stability of samples (Chetwynd et al., 2017).

3.3.3. Other biofluids

Recent research in metabolomics has given increasing attention to saliva, a biofluid obtainable non-invasively and with a relatively low cost. Saliva provides an easy access to the metabolites secreted by the human body (Kelly et al., 2011). It can be obtained by stimulation with citric acid, or without stimulation (resting). Previous studies suggest that almost all metabolites are higher in unstimulated saliva when compared to the stimulated saliva (Takeda et al., 2009). Therefore,

most groups choose to use unstimulated saliva samples, usually following a period of fasting and delayed oral hygiene to prevent contamination (Chetwynd et al., 2017). Typically, saliva samples are collected, centrifuged to eliminate cellular and food debris, and then immediately stored at −80 °C until analysis (Álvarez-Sánchez et al., 2012). Storage at −20 °C can be performed for up to 3 weeks with no detrimental effects (Takeda et al., 2009). To our knowledge, saliva metabolomics has not been yet applied to the study of retinal diseases. However, salivary metabolic profiling in conjunction with serum based metabolic profiling was able to provide diagnostic biomarkers of neurodegenerative dementia, such as Alzheimer's disease and frontotemporal lobe dementia (Mal, 2016).

Another biofluid that can be obtained non-invasively is tears. Human tears have been used to identify potential biomarkers of ocular surface diseases (Nishtala et al., 2016; Zhou et al., 2006; Zhou and Beuerman, 2012). However, recent work suggests that this biofluid also has potential to the study of posterior segment conditions, in particular diabetic retinopathy, (Ting et al., 2016), and also of non-ophthalmologic systemic diseases (Pieragostino et al., 2015). Two main methods have been described for tear collection for metabolomic profiling: the use of Schirmer's strips and the use of glass capillary tubes. Schirmer's strips are routinely used in ophthalmology and enable a simple procedure. Collection should be performed without anesthesia, and then the strips should be stored at −80 °C in glass vials (Lam et al., 2014). The main disadvantage relates to the risk of cellular contamination with epithelial cells (Chen et al., 2011). Glass capillary collection has minimal cells contamination risk, but is a more complex procedure. Previous authors described collection from the lower conjunctival sac with glass micropipettes, followed by cooling to 4 °C and centrifugation to remove possible cellular debris. The resultant supernatant was then stored at −20 °C (Rantamäki et al., 2011) or −80 °C (Lam et al., 2014).

For the study of vitreoretinal diseases, it is also important to consider using aqueous humor. Changes in the composition of this biofluid have been described in patients with age-related macular degeneration and diabetic retinopathy, among other vitreoretinal conditions (Pietrowska et al., 2018; Kersten et al., 2018). The collection of aqueous humor requires performing an anterior chamber paracentesis, or obtaining a sample at the time of intra-ocular surgery. Volumes ranging between 50 and 100 µL have been successfully used for untargeted metabolomic profiling (Pietrowska et al., 2018). As for other biospecimens, aqueous humor should be immediately frozen and stored at −80 °C (Pietrowska et al., 2017).

3.3.4. Paraffin-embedded tissue

Tissues in available biobanks have often been preserved with formalin fixation followed by paraffin embedding (FFPE). Although fresh frozen samples are the gold standard for metabolomics, available archival FFPE tissue samples may be a viable alternative (Nirmalan et al., 2008). These tissues have two major challenges: first, fixation in formalin can alter proteins and biomolecules present in the tissue, and secondly, it is hard to subsequently remove the paraffin (water insoluble) without damaging or losing compounds. However, investigators have described successful metabolomics profiling using FFPE tissue, with good correlation with results from fresh-frozen samples (Cacciatore et al., 2017; Wojakowska et al., 2015; Kelly et al., 2011). For example, Wojakowska et al. (2015) reported the applicability of FFPE kidney specimens for non-targeted GS/MS-based profiling. MSI might be particularly well suited for the use of these tissues (Buck et al., 2016). The possibility of conducting metabolomic studies using FFPE material would open a wide range of tissues for clinical research.

3.3.5. Fresh human retinal tissue and vitreous samples

Human tissues are metabolically active and therefore require rapid metabolic quenching immediately after collection. In general, it is recommended to rapidly wash them after collection (in a phosphate-buffered aqueous solution or in saline), and then proceed as soon as possible with freezing, in order to quench metabolism. Washing of tissues to remove as much blood as possible is also an important step, as the blood metabolome is different from the tissue metabolome and would confound the results. Storage should be performed at −80 °C.

Retinal tissue is not an exception. In the 1980s, Schmidt et al. (1980) described that retinas from post-mortem human donor eyes can retain their metabolic activity for 4–4.5 hours, namely for photoreceptor cell-specific metabolic processes. However, to our knowledge, no studies have been performed proposing a specific protocol for human retinal tissue processing for metabolomics. Based on their findings in rat retina tissue, Tan et al.(2016a,b) recommended that post-mortem human tissue should be stabilized within 8 h following enucleation.

Regarding the amount of tissue required, for both untargeted or targeted metabolomic studies, 20–100 mg of tissue is typically required to ensure good coverage of the metabolome (Ammerlaan et al., 2014). This can be challenging for eye tissue, especially for the study of the retina, but authors (Tan et al., 2016a,b) have succeeded using rat retina with a mean weight of 25.4 mg. For the vitreous, Young et al. (2009) described successful results with undiluted samples of 0.1–0.2 mL, but we would advise larger volumes of 0.5–1 mL (Paris et al., 2016). These samples should be transferred to sterile tubes, and frozen immediately until analysis. This is important as time dependent post-mortem biochemical changes have been demonstrated in the vitreous, even though they are relatively slow (Boulagnon et al., 2011; Zilg et al., 2015).

4. Role of biostatistics and data analysis

Overall, in metabolomics, the analytical path from data acquisition to biomarker discovery and to produce biologically meaningful data involves numerous steps, including both statistical and bioinformatic approaches (Barnes et al., 2016a). Investigators should consult with a biostatistician before initiating a metabolomics study in order to develop an appropriate experimental design, incorporate proper data cleaning, and create an analytical plan for the metabolomic data. During the study, as mentioned, it is also essential to record all the steps taken in the design of the experiment, sample collection, storage and processing. It is also important to be aware of the strengths and weaknesses of the analytical platform, the pre-processing of the data and the statistical and pathway tools used to interpret the data.

4.1. Main statistical approaches

As described, untargeted metabolomic studies are currently the most popular, and are characterized by the simultaneous measurement of a large number of metabolites on each sample. This strategy, known as top-down strategy, avoids the need for an *a priori* hypothesis on a particular set of metabolites and, instead, considers the global metabolome. Consequently, these studies are characterized by the generation of a diverse array of metabolites with a range of chemical properties (Alonso et al., 2015). Targeted metabolomic studies are hypotheses-driven experiments, and are characterized by the measurement of predefined sets of metabolites, most often in a specific metabolic pathway, with absolute quantification, which results in a high level of precision and accuracy (Putri et al., 2013).

Alonso et al. (2015) described with great detail the typical methodological pipeline of an untargeted metabolomic study. Briefly, the first step is the processing of spectral data to generate metabolomic information (i.e. metabolic features), which is highly dependent on the analytical technique used (for example, NMR or LC-MS). Once the complete set of metabolomic features has been generated and run through a quality control pipeline, univariate and multivariate statistics can be applied to determine how the metabolomic features are related

to each other and to the phenotypes of interest. Bioinformatic approaches can then be used to assess the metabolic pathways that are related to the phenotype. In this manuscript, we aim to summarize the main statistical techniques available for data analysis.

After metabolomic data are generated, the distribution of each metabolite is most often largely skewed. This is partially biological in nature, but also due to limitations of the current available analytical platforms (i.e. it can happen when actual values are below the detection limits for a given method). Based on the assumption that most missingness for a given platform is due to limits of detection, options to deal with this include: imputing values that are a fraction (for example, 0.5) of the lowest value measured for a given metabolite; replacing all missing values with zero; and, if a substantial proportion of metabolites have missing data, excluding them, or treating them as dichotomous variables (non-missing *versus* missing). Of note, all these approaches may introduce bias. After addressing missingness, metabolite variables typically benefit from transformation and scaling to allow accurate statistical analyses. A common approach is to log-scale the data, as it has been observed that metabolite concentrations are more often close to log-normal distributions than to normal distributions, but other options are also available (Suhre et al., 2011). Additionally, it is important to always carefully assess batch-to-batch variability. Ideally, all samples in a study should be analyzed in the same batch (i.e. should be extracted and measured in one uninterrupted sequence). When this is not possible, a plan should be established with the laboratory performing the analyses (including randomization of sample order), but batch effects can often persist even after preprocessing steps have been successfully completed. Normalization of data to internal standards or to pooled plasma measures are options to deal with this problem, as well as considering the time of analysis as a covariate (Wehrens et al., 2016; Rusilowicz et al., 2016).

When a quality pipeline has been run, univariate analysis methods usually follow, to perform an initial evaluation of the basic relationships between each metabolite and the phenotype of interest (Barnes et al., 2016b). However, more complex analyses are necessary in order to comprehensively analyze the data, namely to account for important covariates and possible interactions. The metabolomic data obtained from biological samples is often very complex with the presence of correlations between features from the same metabolite and correlations among metabolites from the same pathway. Also, the effect of potential confounding variables like gender, diet, or body mass index is not taken into account by univariate methods, increasing the probability of obtaining false positive or false negative results (Alonso et al., 2015).

Another approach is to evaluate the relationship between a group of metabolites, and thus analyze metabolomic features simultaneously. In this case, both unsupervised and supervised techniques are utilized.

Unsupervised techniques do not use any phenotypic classifications that are specific to the dataset, which means that, for example, in a study comparing samples from patients with a certain disease with a control group, with this approach one does not inform the software which samples belong to each group (Barnes et al., 2016b). This provides an effective way to detect data patterns that are correlated with biological variables (Alonso et al., 2015). One of the most commonly used unsupervised techniques is principal component analysis (PCA). PCA is based on the linear transformation of the metabolic features into a set of linearly uncorrelated (i.e., orthogonal) variables known as principal components, which can be used as independent variables. By plotting the scores (the weighted sum of the contribution of each metabolite to a principal component) of these components, it is possible to find if a group of samples is distinct from one another – Fig. 2. This decomposition method maximizes the variance explained by the first component, while the subsequent components explain increasingly reduced amounts of variance. The first principal components usually capture most of the variability in the dataset. PCA is also used in metabolomics studies to assess data quality, since it can identify sample outliers or reveal hidden biases in the study (Yin et al., 2013).

In supervised techniques, the phenotypic information is used in conjunction with the metabolomic data. Using the same example described above, in the study comparing samples from patients with a certain disease with a control group, with this approach one would inform the software which samples belong to each group. One of the most commonly performed techniques is partial least squares (PLS). Unlike PCA, PLS does not maximize the explained dataset variance but rather the covariance between the variable of interest and the metabolomics data. Therefore, the feature coefficients (loadings) of PLS components represent a measure of how much a feature contributes to the discrimination of the different sample groups (Alonso et al., 2015). The major disadvantage of this approach is that some metabolic features that are not correlated with the variable of interest can actually influence the results and are missed. Orthogonal PLS is a derivation of PLS and has been developed to deal with this limitation (Bujak et al., 2016).

Supervised techniques, such as partial PLS-discriminant analysis (PLS-DA, Fig. 2), are usually preferred to identify new metabolomic biomarkers. Their usefulness, however, must be evaluated, as a significant difference in the average levels of metabolites between two patient groups does not necessarily mean that the given compound will be a good classifier/biomarker (Xia et al., 2013). Indeed, PLS-DA has the risk of overestimating group separation, thus its R^2 (degree of fit to the data) and Q^2 (quality assessment) parameters should be assessed. These parameters, however, have limitations and are strongly application dependent. An invalid or irrelevant model can still produce good values, but empirically the acceptable value for Q^2 is $\geq 0.4/0.5$. Highly disparate R^2 and Q^2 values are an indicator of possible model overfitting (Szymańska et al., 2012; Triba et al., 2015). To prevent overfitting of the models, the number of variables can also be reduced. Apart from PLS-based techniques, this can be achieved by using the Least Absolute Shrinkage and Selection Operator (LASSO) (Bujak et al., 2016) or Ridge Regression methods (Acharjee et al., 2012).

As in most other biomedical fields, receiving operator characteristic (ROC) curve analysis is generally considered a standard method for performance assessment (Obuchowski et al., 2004). A ROC curve is a non-parametric (i.e. not dependent on data normality) measure of biomarker utility (Xia et al., 2013), that compares specificity against sensitivity according to a specific decision boundary – Fig. 3. They are usually summarized with the area under the curve (AUC), which gives the probability that a classifier will rank a randomly chosen positive sample higher than a randomly chosen negative one. A rough guide for assessing the utility of a biomarker based on its AUC is as follows: 0.9–1.0 = excellent; 0.8–0.9 = good; 0.7–0.8 = fair; 0.6–0.7 = poor; 0.5–0.6 = fail (Xia et al., 2013). Typically biomarker discovery studies are relatively small ($n < 100$) when compared to the size of the proposed target population (potentially millions of subjects). As such, any performance measure is a sample approximation to the (unmeasurable) performance of the biomarker applied to the target population as a whole. Therefore, confidence intervals (CIs) should always be reported.

It is also imperative that, regardless of the reported predictive indices, the performance of any biomarker is validated. The easiest approach for cross-validation is to split the dataset into two parts, the training set and the validation set. The model optimization should be performed on the training subset, and then model performance is assessed on the validation set. Several rounds should be performed (Xia et al., 2013). However, the most robust measure of validation is replication in an entirely independent cohort. Following this, targeted metabolomics and studies to assess the biological mechanisms are also recommended.

In this section, we summarized the statistical approaches that, to date, are the most commonly used for metabolomics data analysis. However, machine learning methods have also been applied to different steps of modelling for these data (Cuperlovic-Culf, 2018). These methods offer advantages in dealing with the strong correlations

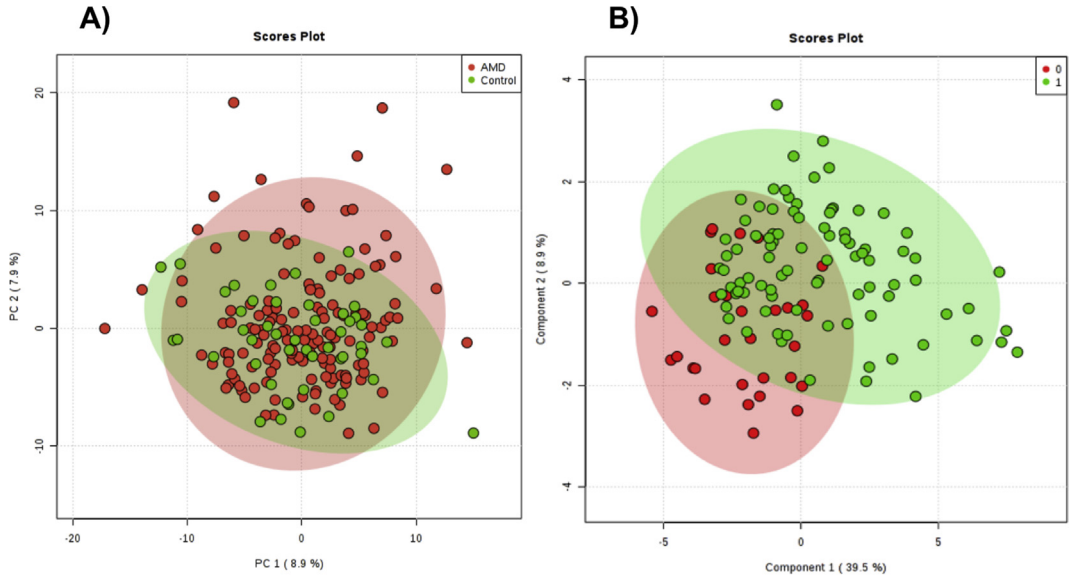

Fig. 2. Example of principal component analysis (PCA) (A) and partial least square discriminant analysis (PLS-DA) score plots (B). (A): PC – principal component; in the graph, the x-axis corresponds to PC1 and the y-axis to PC2; (B): in the graph, the x-axis corresponds to component 1 and the y-axis to component 2. In both (A) and in (B), red dots correspond to patients with age-related macular degeneration (AMD) and green to control subjects.

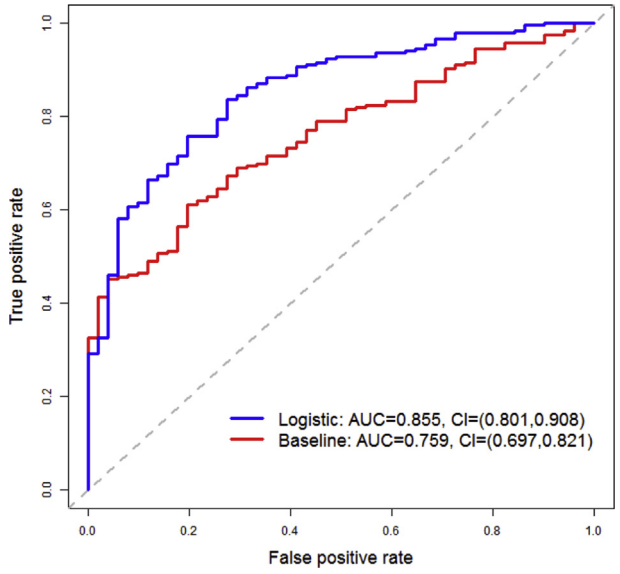

Fig. 3. Example of a receiving operator curve (ROC) analysis. Data refers to plasma samples of a cohort studied by our group (unpublished data) with 242 patients with age-related macular degeneration and 53 controls. In red, a baseline model including only clinical covariates (i.e. age, gender, smoking status and body mass index); in blue, metabolites data is additionally considered. As shown, the model including metabolomics data significantly outperformed ($p = 0.003$) the model only with clinical covariates. AUC – area under the curve; CI – confidence interval.

existing among metabolites, as multiple signals can belong to the same metabolites, and metabolic connections can exist along physiological pathways (Blaise et al., 2010). In the near future, it is likely that machine learning methods assume a more prominent role.

4.2. Sample size and power

In research, sample size determination is a critical part of study design (Chi et al., 2014), and it is determined utilizing statistical power calculations. Power is the probability of rejecting the null hypothesis when one is truly there (Billoir et al., 2015). In medical studies, researchers often have to balance sufficient statistical power with the costs involved in a project. If a study is underpowered, the risk that important findings are missed increases, thus resulting in a waste of time and money. Alternatively, if a study is oversized, the overall cost of the study increases.

In metabolomic studies, many factors complicate sample size determination, thus requiring adapted tools. The metabolome is considered to be more diverse and thus more complex than other ensembles. As detailed, metabolites do not operate in isolation but through a complex network of interactions (Blaise et al., 2010). Most of the conventional methods developed for sample size determination are based on the principle of variable independence, which is obviously not appropriate for metabolomic studies (Billoir et al., 2015). Finally, like the remaining 'omics, in metabolomics we also have to face constraints connected to multiple hypothesis testing, as discussed below.

A recent report by the training workshop group on metabolomics, supported by the NIH Common Fund Program in Metabolomics, has highlighted a few general points to consider (Barnes et al., 2016a). According to these authors, for studies with laboratory animals on controlled diets, a sample size of 6–12 animals may be adequate, although if female animals undergoing estrus cycling are used, they should be studied during the same point in the estrus cycle. Controlled clinical studies where subjects provide multiple samples or where the subjects are carefully matched may be possible to be carried out with as few as 10–20 patients, but this will depend on the variance of the disease traits, drug response or that which is introduced by an interventional procedure. These sample numbers (n = 3–20) are suitable for generating preliminary and/or pilot data. For epidemiological studies, where the samples are collected from a general population, often over long periods of time, variance is a substantial issue and may require patient numbers in the thousands (Barnes et al., 2016a). In the last few years, important research has addressed the challenge of merging data from different large scale studies and biobanks (Chi et al., 2014; Dane et al., 2014; Dunn et al., 2011a).

Recently, online platforms, such as MetaboAnalyst 3.0 (Xia et al., 2015) or metaX (Wen et al., 2017), developed modules that enable a precise sample size estimation and power analysis for designing metabolomic studies. In both cases, the available software relies on the Bioconductor Package SSPA that was originally developed for genomic data (van Iterson et al., 2009). This method requires the use of pilot

Table 1
Selected literature on the application of metabolomics to the study of retinal diseases.

Condition	Authors	Year	Specific focus	Type of samples analyzed	Analytical tool	Sample size
Diabetic retinopathy	Barba et al.	2010	PDR	human vitreous	1H-NMR	22 per group
	Paris et al.	2016	PDR	human vitreous and oxigen-induced retinopathy mouse model (whole eye)	LC-MS	9 to 20 per group
	Chen et al.	2016	Diabetes with and without DR	human plasma	GC-MS	40 per group
	Li et al.	2011	DR Western and Chinese medicine	human plasma	GC-MS	25 to 50 per group
Retinal detachment	Li et al.	2014	RRD and PVR	human vitreous	LC-MS	6 to 9 per group
	Yu et al.	2015	RRD and choroidal detachment	human vitreous	LC-MS	14 to 15 per group
AMD	Osborn et al.	2013	Choroidal neovascularization	human plasma	LC-MS	19 to 26 per group
	Laíns et al.	2017	AMD and AMD severity stages	human plasma	1H-NMR	30 to 45 per group
	Laíns et al.	2017	AMD and AMD severity stages	human plasma	LC-MS	30 per group
	Li et al.	2016	Polypoidal choroidal vasculopathy	human plasma	LC-MS	19 to 21 per group
	Thomson et al.	2015	Sub-RPE deposits formation and composition	sub-RPE deposits from human donor eyes	SIMS	30 (total), 6 with soft drusen and basal deposits and 10 with hard drusen
	Rowan et al.	2017	AMD, diet influence and microbiome	plasma, urine and feces of aged-mouse model	LC-MS plasma and 1H-NMR urine	4 to 11 per group
Macular telangiectasia	Scerri et al.	2017	MacTel type 2	human serum	LC-MS	50 per group
Others	Young et al.	2009	Vitreoretinal disorders (mostly uveitis)	human vitreous	1H-NMR	2 to 20 per group
	D'alessandro et al.	2014	Retinal ischemia and effect of neuroprotectors	*ex vivo* mouse retinas (whole retina) deprived oxygen	LC-MS	5 per group
	Kurihara et al.	2016	Hypoxia induced stress in RPE	mice choroid/RPE complex (non-retinal)	LC-MS	4-6 per genotype
	Luan et al.	2018	Hibernating retina	hibernating squirrels	GC and LC-MS	6 per condition
	Solberg et al.	2013	Postnatal hypoxia	piglet retina after hypoxia	LC-MS	5 per condition

AMD – age-related macular degeneration; PDR – proliferative diabetic retinopathy; DR- diabetic retinopathy; RRD – rhegmatogenous retinal detachment; RPE – retinal pigment epithelium; MacTel – macular telangiectasia; NMR – nuclear magnetic resonance; GC – gas chromatography mass spectrometry; LC – liquid chromatography mass spectrometry; SIMS - secondary ion mass spectrometry.

data. Briefly, users first need to upload their pilot metabolomic data and perform the conventional data processing and normalization steps. Several diagnostic plots are then presented to allow users to check whether the test statistics follow an approximately normal distribution, and whether there are relatively a large number of P values that are close to zero (i.e. the effect indeed exists). When these assumptions are reasonably met, users can proceed to estimate the statistical power with regard to different sample sizes.

Considering that pilot data is often not available, another option is MetSizeR (Nyamundanda et al., 2013), which is based on the idea that the method for selecting sample size firmly depends on the type of data analysis the researcher intends to employ. In a situation where experimental pilot data are not available, pseudo-metabolomic data can be simulated from a statistical model. The specific statistical model from which the pseudo-metabolomic data are simulated depends on the type of statistical analysis that the metabolomic scientist intends to use (such as, for example, principal component analysis). The main criticism of this approach is that experimental conditions (including sample preparation, storage, data acquisition) should be identical in the simulated data and the planned experiments, which is difficult to account for. Therefore, some authors argue that pilot studies might be inevitable despite their cost, and that inadequate sample size estimations would raise more ethical issues and costs than a properly designed pilot study (Billoir et al., 2015).

4.3. Multiple hypothesis testing

Like the remaining 'omics sciences, metabolomics deals with concerns related to multiple hypothesis testing, which increase the risk of false discovery (type I errors, false positives). Numerous methods have been developed to control for multiple testing in the genomics and transcriptomics fields. The most well established are family-wise error risk (FWER) controls, such as the Bonferroni correction; and false discovery rate (FDR) measurements, such as the Benjamin-type corrections (Billoir et al., 2015). The FWER estimates the number of variables associated with a true null hypothesis and that are proposed as significant by any given statistical test. The FDR controls the rate at which variables identified as significant by a given test are in fact associated with a true null hypothesis (Billoir et al., 2015). The FDR correction of the p-value results in a q value (Barnes et al., 2016b).

In metabolomics, the optimal method is still under debate. However, in general, it is recognized that the FWER corrections are too conservative, and FDR measurements are more suited and thus are becoming widely accepted (Billoir et al., 2015; Barnes et al., 2016b; Alonso et al., 2015). FDR is a less stringent, multiple testing correction that results in fewer features/metabolites as false negatives (Barnes et al., 2016b).

4.4. Pathway analysis

One of the most important advantages of metabolomics is the ability to provide meaningful biological contexts for the data generated. However, this is challenging and much less straight-forward than for genomic and proteomic datasets. Enrichment tools are frequently used, as they include molecular pathway or network information to gain insight into a biological system, mostly by performing functional enrichment or over-representation analysis. In practice, these approaches assess, respectively, if metabolomic pathways differ in the experimental dataset of interest *versus* control datasets, and whether specific pathways containing metabolites in an experiment derived list are over-represented (Rosato et al., 2018).

Several enrichment tools are available, including MetaboAnalyst (Chong et al., 2018), IMPaLA (Kamburov et al., 2011) and BioCyc/HumanCyc (Romero et al., 2005), among many others (Marco-Ramell et al., 2018). These tools enable to visualize the localization of metabolites in a certain pathway, their molecular connections, reactions and relational networks. Interestingly, as recently reviewed by Marco-Ramell et al. (2018), even though the tools differ, they perform consistently. Nevertheless, there are limitations. For instance, most of these platforms function on the basis of metabolite name or codes (identifiers) from one or several metabolite database, and current metabolite databases are limited (Kell and Oliver, 2016), as discussed in our section "7. Future Directions". If possible, more than one tool should be used.

5. Metabolomics of vitreoretinal health and disease

Metabolomics has been applied by different groups to the study of normal vitreous and retina, as well as to the study of several pathologic conditions. Due to the versatility of metabolomics, these include: clinical applications aiming to identify biomarkers of retinal diseases; attempts to identify novel mechanisms behind the development of certain conditions, in order to discover new potential targets for future research; and clarification of mechanisms identified by other research techniques.

In this section, we critically review relevant work performed in this field (normal and pathology). Table 1 summarizes data on the study design of selected literature applying metabolomics to the study of retinal diseases.

5.1. Normal vitreoretinal metabolome – animal model investigations

5.1.1. Normal retina

Understanding the classes of common metabolites that can be expected in normal retina tissue is important, as it serves as baseline knowledge to recognize and comprehend pathological states. By generating a complete metabolomic profile, we can increase our understanding of the potentially multifaceted roles of the different molecules in regulating and maintaining retinal development, health, and function. To our knowledge, however, studies of normal retina tissue are currently limited to animal models.

Using GC-MS and LC-MS, Du et al. (2016) assessed the metabolomic profile of normal whole mouse retina tissue. Targeted metabolomics of 171 metabolites was performed, and data were obtained for 114 of them. These included metabolites involved in glucose metabolism (anabolism and catabolism), tricarboxylic acid cycle (TCA) cycle, amino acids, nucleotides and their metabolites, tryptophan cycle metabolites, vitamins, and a small number of sugars and lipids/fatty acids. Tan et al. (2016a,b) studied the neurosensory rat retinal metabolome, and compared it with the metabolite composition of the remaining rat ocular tissues (cornea, lens and vitreous). Interestingly, the authors found that the retina displayed the most unique profile of identified metabolites, with 655 detected only in this tissue. In total, 21 metabolites were identified in rat retina tissue with GC-MS and 1942 with UHPLC-MS, belonging to many different classes. Importantly, other groups described that the rat retinal metabolome varies with sex (male vs female), mostly in terms of glycerophospholipids, biogenic amines and amino acids metabolites (de la Barca et al., 2017). This is important for the design of future studies using animal retina tissue.

Imaging MS (MALDI-FTICR-MS) of retina tissue has also been described (Sun et al., 2014). Using adult porcine eyes, Sun et al. were able to generate segmentation maps, which were then used to identify areas in which similar spectra occurred across the tissue samples. Twenty-three different metabolites were identified in situ, including nucleotides, glucose-6-phosphate and other metabolites belonging to the central carbon metabolism pathway, and lipids, among others. This was the first study to utilize FTICR-MS, but prior studies with MALDI-TOF had also described distributions of different phospholipids and fatty acids in the different retinal layers (Ly et al., 2015; Zemski Berry et al., 2014).

In addition to the characterization of the global metabolomic profile of the retina, metabolomics has also contributed to the understanding

of retinal physiology. The retina has an extremely complex anatomy and physiology, and its main function is to transduce light stimuli into chemical signals through the process of phototransduction (Luo et al., 2008). This process requires high amounts of energy (Wong-Riley, 2010). In the outer retina, where the photoreceptors are located, the consumption of oxygen, as well as of glucose, is higher in the dark than in the light (Linton et al., 2010); in the inner retina, their consumption seems to be more independent of light exposure (Lau and Linsenmeier, 2012). This happens because, in response to light stimulation, photoreceptors hyperpolarize, and cease neurotransmitter release, thereby decreasing their metabolic demands (Wong-Riley, 2010).

Du et al. (2016) compared the levels of metabolites from dark-adapted mouse retinas to retinas from mice exposed to light. Their results revealed that with illumination, among other changes, the levels of cGMP were depleted by 55%, probably due to increased hydrolysis by phosphodiesterases; and that inosine monophosphate (IMP) accumulates. Most other purine and pyrimidine metabolites, which seem to be derived from photoreceptors, are however depleted. The authors suggested that the observed increase in nucleotide levels in darkness may be linked to: degradation of RNA; accumulation of products from cGMP degradation (such as 5'-GMP) that inhibit *de novo* purine synthesis; or block of purine synthesis via the salvage pathway. The influence of illumination on nucleotide metabolites was a new observation that may have important implications for the study of retinal diseases, namely for understanding why retinas are uniquely sensitive to certain enzyme deficiencies and not to others.

Imaging MS of adult porcine retina tissue (Sun et al., 2014) also showed a number of notable differences in the distribution profiles of the detected metabolites between light and dark-treated tissue samples, particularly those from the glycolysis pathway. As noted above, the metabolism of the outer retina is higher in the dark and reduced with light (Wong-Riley, 2010), which seems to be confirmed by this metabolomic technique. With light, the authors described a shift of glycolysis' products, such as fructose 1,6 bisphosphate (F1,6BP; a product of glycolysis) and citrate, to the inner retina, while glucose 6 phosphate (G6P) was detected at high intensities both in the inner and outer retina. This is probably due to a slowing of glycolytic activity in the outer retina. The outer retina is avascular and is nourished by the choroid via the RPE, which is not altered by light stimulation. Therefore, while the delivery rate of glucose and glucose phosphorylation remains the same as in dark conditions, decreased glycolytic activity would result in decreased levels of downstream metabolites following exposure to light. In general, there were also relatively higher amounts of all metabolites under dark conditions, which probably reflects the function of photoreceptors and their specialized metabolic requirements (Sun et al., 2014). Understanding the distribution of metabolites in the retina and their differences in light and dark conditions might be important to understand retinal diseases.

Interestingly, De La Barca et al. (2017) recently studied the effect of light stress in the retina metabolome. This was based on the work showing that the mechanisms of light preconditioning (pre-exposure to moderate light before intense light) remained unknown, even though they seemed to have a protective effect. The authors observed that light stimulation induced changes in lipid and amino acid metabolism, with a likely involvement of nitric oxide-related signaling pathways.

With aging, the metabolomic profile of the retina may also change. Using targeted MS applied to an aging mouse model, Hopiavuori et al. (2017) studied the changes in whole retina and brain composition of the three major glycerophospholipid classes: phosphatidylcholine (PC), phosphatidylethanolamine (PE) and phosphatidylserine (PS). The authors described that retinal PC contained detectable levels of very-long chain polyunsaturated fatty acids (PUFA), which were not observed in brain tissue, and remained constant with age. However, like in the brain, an age-related reduction of PE and PS, as well as some other PCs, was observed in the retina. The authors hypothesized that these age-related changes may have profound effects on synaptic function and cognitive ability. In the same study, it was also described that the retina is unique because PC, PS and PE contain a greater amount of di-PUFA species than the brain, and that plasmalogens (PE) are present but in lower levels. Plasmalogens are important for membrane biophysical properties and neurotransmission, as well as for protection in response to cellular oxidative stress. Gordon et al. performed a mini-review proposing potential targets for lipidomics studies of retina tissue, namely to better understand the mechanisms of inflammation, neuroprotection and nerve regeneration (Gordon and Bazan, 2013).

5.1.2. Normal vitreous

The vitreous is the largest structure of the eye, occupying the posterior segment, between the lens and the retina. Although in forensic science the vitreous has long been considered crucial in identifying certain causes of death and to estimate the time of death, (Zilg et al., 2015; Boulagnon et al., 2011) in ophthalmology the vitreous was considered relatively irrelevant until recently. This has changed dramatically in the last few decades, with the vitreous being recognized as an important factor in ocular health and disease, including in the pathogenesis of retinal detachment and macular hole formation, and of diabetic retinopathy (Holekamp, 2010). In addition to its role in maintaining a normal interface with the retina, the vitreous is also now thought to be involved in normal oxygen metabolism and consumption. Shui et al. (2009) suggested that the vitreous may protect the lens and the trabecular meshwork from oxidative stress, by metabolizing the oxygen diffused from the retina into ascorbate. This function seems to deteriorate with vitreous age-related liquefaction. The ability of the vitreous to regulate intra-ocular oxygen tension has also been hypothesized to affect VEGF-mediated diseases. However, data on the clinical relevance of this hypothesis remain controversial (Cuilla et al., 2015; Singh et al., 2017).

Using a 1H-NMR untargeted approach, Locci et al. (2014) provided interesting inputs into oxygen vitreous metabolism. The authors used vitreous samples from goat, and studied the vitreous gel as a whole, as well as four distinct areas – cortical, core, and superior and inferior basal vitreous. A unique metabolomic signature was observed for each area. The vitreous base (basal vitreous adjacent to the lens and the trabecular meshwork) was characterized by the presence of branched-chain amino acids (BCAA), betaine, alanine, lysine, myo-inositol and ascorbate. The presence of ascorbate in this area is in agreement with the theory that this molecule has a differential expression in the vitreous, protecting the lens and trabecular meshwork from oxidative stress (Shui et al., 2009). Betaine and myo-inositol probably serve as osmoregulators, which is important to maintain retinal structure and function. BCAA may represent an alternative energetic source to glucose, which is critical in areas with higher metabolic demands, such as the cortex (Locci et al., 2014).

In the cortical area, the discriminating metabolites included glutamine, choline and its derivatives, N-acetyl groups, creatine, and glycerol. However, the most abundant metabolite was lactate (Locci et al., 2014). This suggests that the cortex is the most metabolically active area, relying on a fast glucose-driven metabolic response and its anaerobic pathway since the lactate is the product of anaerobic glycolysis within the eye, when there is limited availability of oxygen. Rucker et al. (2003) also described that lactate was the dominant resonance in the human vitreous spectra, using proton NMR; the authors suggested that lactate could be used as a molecular marker to evaluate retinal and optic nerve metabolism.

The presence of glutamine points to a role of cortical vitreous in preventing neurotoxicity. Glutamate is the main neurotransmitter in the retina, and its toxicity is avoided by converting it into glutamine (Zeng et al., 2010). The vitreous core revealed the presence of glucose, acetate and scyllo-inositol (Locci et al., 2014); this led the authors to hypothesize that this is due to a passive diffusion of these energetic molecules through the vitreous to make them available for the more active areas near the retina.

Despite the evidence suggesting metabolic interactions between the vitreous and the retina, using a rat model, Tan et al. (2016a,b) described that only a small percentage (1.6%) of the metabolites identified in the vitreous using MS, were exclusively found in the retina and not in other tissues. The authors suggested that the metabolic exchange/movement between the cornea and aqueous, and the lens with vitreous and aqueous may explain the similarities between the metabolites found in the lens, vitreous and cornea. However, due to the posterior bulk flow of water from the vitreous through the retina (Zeng et al., 2010), the anterior movement of small molecules from the retina into the vitreous might be inhibited, thus resulting in the vitreous metabolome not reflecting that of the retina. Therefore, even though vitreous has been used as the surrogate for analyzing molecular changes taking place in the retina, this might not always be appropriate, especially in physiological states. In some pathological states, where there is increased permeability of the retinal vessels or retinal pigment epithelium, vitreous analysis may be of value (Tan et al., 2016a,b). Other authors argue however, that exactly because of the existence of the blood-retinal barrier, the vitreous can reflect the intraocular environment effectively (Yu et al., 2015).

Importantly, the described findings for normal metabolomic vitreous composition refer to animal models and they seem to vary among species. For example, Mains et al. (2012) observed clear differences in the metabolomic profiles of vitreous samples from sheep, rabbits and pigs. These were primarily related to the content of DNA and RNA-related metabolites, as well as metabolites associated with the diet.

5.2. Diabetic retinopathy

Diabetic retinopathy (DR) affects 93 million individuals world-wide, and of those, 5 million are completely blind (Stem and Gardner, 2013). Irreversible vision loss occurs in mid-to late-stages of DR as a result of either macular edema (due to disruption of the blood-retina-barrier) or neovascularization (due to ischemia). Current therapies that target vascular complications are only effective in 25–50% of DR patients (Abcouwer and Gardner, 2014), hence there is a real need for new therapies. Metabolic disturbances are a known component in the pathogenesis of diabetes. Thus, metabolomics analysis of human samples may shed light on the mechanisms of DR and identify potential therapeutic targets.

Two studies have used metabolomics analyses on vitreous humor from DR patients, to identify biomarkers of the disease (Barba et al., 2010; Paris et al., 2016). Barba et al. (2010) analyzed vitreous samples from 22 type 1 diabetic patients and 22 controls with macular hole (MH) using an 1H-NMR-based approach. Through partial least square-discriminant analysis (PLS-DA) the authors were able to correctly classify patients and controls based on metabolomics patterns, with a sensitivity of 86% and a specificity of 81%. Notably, they found that acetate, glucose, sorbitol and mannitol levels were elevated in the vitreous of patients with proliferative diabetic retinopathy (PDR), Fig. 4. In contrast, galactitol and ascorbic acid levels were significantly lower in the vitreous of PDR patients when compared to controls. Paris et al. (2016) used global metabolomics to both identify PDR biomarkers and to confirm the relevance of the rodent model of oxygen-induced retinopathy (OIR) as a model of PDR. For their PDR vitreous studies, the authors used two cohorts. Firstly, untargeted metabolomics, was done on a cohort of 9 PDR vitreous samples and 11 non-diabetic controls. Results from this analysis were then validated using targeted metabolomics on a second cohort. In their studies, 129 metabolites were found to be dysregulated. These included allatoin, glutamate, lysine, arginine, N-acetylaspartate, iditol, glycerate and N-acetylglutamte. Validation with targeted analysis confirmed that arginine and allantoin levels are elevated in the vitreous of PDR patients. Next, to confirm the relevance of OIR as a model of PDR, global metabolomics was performed on mouse retinas. Among other dysregulated metabolites, arginine was also increased in OIR retinas, when compared to controls, similar to what is observed in PDR samples.

As discussed, factors limiting the usefulness of using vitreous metabolomics, are the surgical procedure to collect vitreous (vitrectomy), which limits choice and availability of control samples, and the relatively small volume (a maximum of 1 ml) obtained. In contrast, plasma samples are easily collected and in larger volumes. Chen et al. (2016) performed a nested, population-based, case-control study as part of the Singapore Indian Eye Study (SINDI), looking at plasma samples from patients with diabetes type 2: 40 of them with DR (moderate non-proliferative DR), and 40 without signs of DR. The authors observed that DR samples had decreased levels of 1,5-anhydroglucitol and increased levels of 1,5-gluconolactone, 2-deoxyribonic acid, 3,4-dihydroxybutyric acid, erythritol, gluconic acid, lactose/cellobiose, maltose/trehalose, mannose, ribose, and urea. Some of these metabolites, however, lost their statistical significance when accounting for confounding factors. The identified metabolites were then separately quantified in a different cohort from the SINDI study, and increased plasma concentrations of five of them (2-deoxyribonic acid, 3,4-dihydroxybutyric acid, erythritol, gluconic acid and ribose) were seen in patients with DR as compared with subjects with diabetes and without DR signs, thus validating the potential of these biomarkers. Pathway analysis revealed a significant enrichment of the pentose phosphate and galactose metabolism pathways, suggesting the involvement of oxidative stress in the disease.

Li et al. (2011) also successfully used metabolomics analysis for the study of plasma samples of DR patients. Their goal was to stage DR disease progression according to both Western and Chinese medicine standards and to identify potential biomarkers. In their studies, 89 type 2 diabetic patients with or without DR and 30 non-diabetic controls were used. Through orthogonal signal correction-partial least-squares discriminant analysis (OSC-PLSDA), the authors were able to segregate the diabetic samples into three stages: pre-clinical DR, non-proliferative DR and proliferative DR. They were also able to segregate these samples into Yang deficiency and non-Yang deficiency, the two-stages of DR according to Chinese medicine. The authors identified 8 metabolites as potential biomarkers for DR, according to Western medicine. These

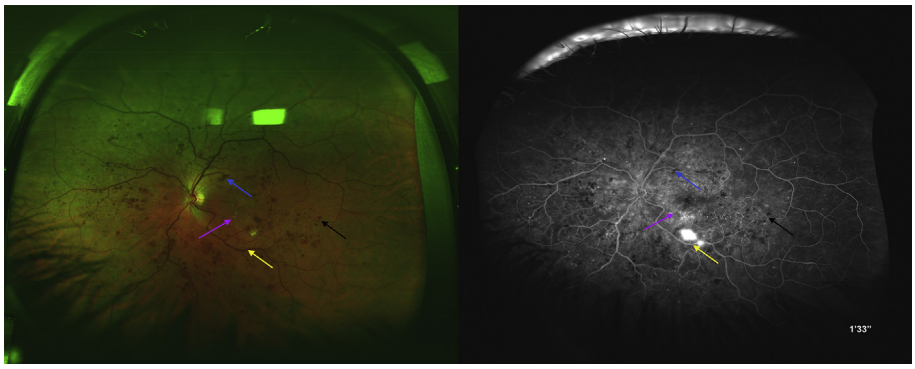

Fig. 4. Representative ultra-widefield fundus photography (A) and fluorescein angiography (B) of a left eye with proliferative diabetic retinopathy. In (A) multiple microaneurysms (black arrow), retinal hemorrhages (blue arrow) and hard exudates (purple arrow) can be appreciated. An area with new vessels (yellow arrow) can also be seen in the inferior temporal arcade, which presents as evident leakage in (B) (yellow arrow).

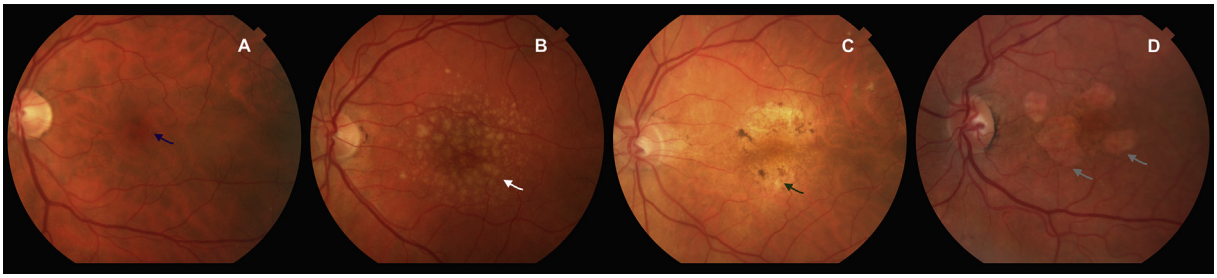

Fig. 5. Color fundus photographs representative of the spectrum of severity stages of age-related macular degeneration (AMD). In (A) the presence of small drusen (blue arrow) and pigment changes in the macular area can be observed – early AMD; in (B), in the macula, the presence of intermediate and large confluent drusen (white arrow) can be appreciated – intermediate AMD; (C) presents an example of an eye with choroidal neovascularization and subsequent fibrosis (green arrow) – late AMD, exudative form; (D) represents an eye with several areas of geographic atrophy (grey arrows) – late AMD, atrophic form.

included, β-hydroxybutyric acid, trans-oleic acid, lineoleic acid and arachidonic acid. Regarding Chinese medicine, a significant decrease in glycerol levels was found in Yang deficiency when compared to non-Yang deficiency.

5.3. Rhegmatogenous retinal detachment

Rhegmatogenous retinal detachment (RRD) is an important cause of vision loss (D'Amico, 2008), afflicting up to 17.9 persons per 100,000 a year (Laatikainen et al., 1985; Mitry et al., 2010; Rowe et al., 1999; Törnquist et al., 1987; Wilkes et al., 1982). Among the potential complications of RRD, the development of proliferative vitreoretinopathy (PVR) is the most common and can lead to irreversible vision loss (Tseng et al., 2004; Pastor et al., 2016). PVR is an irregular scarring process, characterized by the growth of membranes on both surfaces of a detached retina, and on the posterior surface of the vitreous (Ciprian, 2015). Despite the identification of risk factors for the development of RRD, and the recognized role of cellular proliferation, the pathogenesis of this condition remains largely unknown. Additionally, even though several attempts have been made to develop drugs to halt the progression and development of PVR, they have not been successful in humans, and currently there is no accepted treatment besides surgery (Pastor et al., 2016). There is clearly an unmet need to better understand the mechanisms of this disease and to identify novel potential treatment targets (Pastor et al., 2016).

Metabolomics has been used as an approach to better understand these mechanisms. Li et al. (2014) compared the metabolomic profile of human vitreous samples from patients with RRD, with those from patients with recurrent retinal detachment and PVR, along with control specimens from donor eyes. A clear metabolomic separation was observed between the 3 groups, with 31 distinguishing metabolites identified. Eleven metabolites were significantly different between the eyes with RRD and PVR. The findings were interpreted as a dysregulation of pathways related to inflammation, proliferation and energy consumption. Most of the identified metabolites were linked to inflammation, including L-carnitine, which was decreased. Interestingly, the decrease in L-carnitine was significantly more pronounced in eyes with simple RRD than in eyes with PVR. Since L-carnitine inhibits inflammation, this suggests that inflammation is more pronounced in eyes with RRD. Conversely, in eyes with PVR, metabolites such as ascorbate and valine, which have been linked to fibroblast proliferation, seemed to be more prominent. However, another metabolite associated with proliferation, urea, was decreased in PVR, which might represent a downregulation to inhibit proliferation. In the same study, an increase in citrate, succinate and d-glucuronolactone was observed in both RRD and PVR eyes, suggesting abnormalities in energy metabolism, namely in the TCA cycle. This study has important limitations, namely a very small sample size (8 patients with RRD, 7 with PVR and 6 normal) and the lack of a validation cohort. However, it suggests the potential role of metabolomics in delineating the pathophysiology of PVR. The authors did not assess if the inclusion of metabolomics data on clinical predictive models can improve their accuracy, which would be interesting to perform as clinical models alone do not provide sufficient predictive power to identify patients at high risk of PVR (Pastor et al., 2016).

Another group focused on RRD associated with choroidal detachment, another important cause of retinal detachment repair failure, whose pathogenesis remains to be understood (Yu et al., 2015). The authors compared the metabolomic profile of human vitreous samples obtained from patients with isolated RRD, with samples from patients with RRD and simultaneous choroidal detachment (RRDCD). In both groups, the mean time from diagnosis was 4 days. Both on PCA and PLS-DA the authors observed a separation between RRD and RRDCD profiles, which included 265 metabolites, but only 24 were identified. The authors acknowledged that the many unidentified metabolites represent a limitation of their work, as it is likely that metabolomic changes and pathways were missed. Interestingly, most of the metabolites and pathways identified in this study were similar to those identified by Li et al. (2014) distinguishing RRD and PVR, with most metabolites related to the urea and TCA cycle. In eyes with RRDCD, there were increased levels of TCA metabolites, suggesting a higher level of energy metabolism, as well as proliferation-related and inflammation-related metabolites (even though both study groups had the same grade of PVR).

5.4. Age-related macular degeneration

Age-related macular degeneration (AMD) is the leading cause of blindness in people older than 50 years in developed countries, and the third worldwide (Wong et al., 2014). Approximately 90% of patients with AMD have early or intermediate forms (Fig. 5), which may progress to advanced disease, in the form of either geographic atrophy and/or neovascular AMD (also known as “wet AMD”) (Sobrin and Seddon, 2013; Yonekawa et al., 2015). AMD is mostly asymptomatic in its early stages, thus its diagnosis tends to occur only when patients seek a routine eye examination, or are imaged with retinal color photography or optical coherence tomography. Thus, the condition often remains undetected until it is more advanced with loss of vision. Additionally, only advanced neovascular AMD can be treated; despite all research and recent trials, limited options are available to reduce AMD progression, and no treatments exist for the advanced atrophic forms of the disease (Assel et al., 2018). This is at least partially linked to the current gap in our understanding on how genes and environmental factors interact to lead to AMD occurrence and progression, and the current lack of treatments for early disease. Increasing our molecular comprehension of the biological pathways, as well as the specificity of genetic and environmental interactions on AMD, is crucial to develop new treatments and improve patient care (Lorés-Motta et al., 2018). Metabolomics has the potential to (i) increase the current understanding on AMD pathological mechanisms and thus lead to the identification of new therapeutic targets; (ii) identify potential biofluid

biomarkers for screening and assessment of progression; (iii) better characterizing and identifying subtypes of the disease and enable the development of personalized medicine.

Kersten et al. (2018) recently performed a comprehensive review on the utility of several biofluid compounds as biomarker candidates for AMD. The authors concluded that the most promising biomarker candidates were related to oxidative stress pathways, the complement system and lipid metabolism. However, they highlighted that, in general, there was inconsistency among different studies evaluating biomarkers and their association with AMD. The great promise of hypothesis-free techniques, such as metabolomics, was also emphasized.

Osborn et al. (2013) pioneered the application of metabolomics to the identification of potential biomarkers of AMD, by comparing the metabolomic profile of plasma samples of patients with neovascular AMD with controls. Using LC-MS, the authors described 94 metabolites separating the two groups, 86 of them identified as known metabolites. However, they acknowledged that several nucleotides, sugars and lipids, among others were undetectable in their study. Among the 94 metabolites identified, 40 of them were consistently different between patients with neovascular AMD and controls regardless of the statistical approach used. The most significant and relevant metabolites for the separation between the groups were peptides and modified amino acids (increased in patients with neovascular AMD), natural products and environmental agents. Other less prominent features included bile acids (decreased in patients with neovascular AMD) and vitamin D-related metabolites (decreased in patients with neovascular AMD). The authors also performed pathway analysis, and described that the identified metabolites mapped to 17 pathways, mostly related to carbohydrate, amino acid, and coenzyme metabolites required for nitrogen balance and energy metabolism.

Our group was the first to study the metabolomic profile of the different stages of AMD (early, intermediate and late), which we compared to control subjects older than 50 years and with normal macula. Our initial work (Laíns et al., 2017a) was performed with NMR metabolomics, based on the principal that this can be an appropriate technique for an initial untargeted approach. In this study, we included a total of 396 subjects, 61% (n = 243) from Coimbra, Portugal (42 controls and 201 patients with AMD) and the remaining from Boston, United States (n = 153) (40 controls and 113 patients with AMD). Data from both cohorts were analyzed separately. Using variable selection (a technique that enables to filter off random variability unrelated to sample classes (Diaz et al., 2013)), it was possible to observe a separation between multiple AMD stages for the Boston cohort, and between extreme stages (late AMD vs controls and late AMD vs early AMD) for the Coimbra cohort. This separation was mostly due to amino acids and organic acids, dimethyl sulfone, lipids and proteins. The potential confounding effects of gender, smoking history and age on these results were found to be negligible. Interestingly, the metabolomic fingerprints of AMD in the two cohorts presented both similarities and differences. We observed similarities in the variations of histidine, unsaturated fatty acids and protein levels, which suggests that such variations may be a universal reflection of the disease, and, therefore, with potential value in contributing to the current knowledge of the pathogenesis of AMD. On the other hand, cohort differences in relation to variations in particular compounds may reflect the potential importance of local diet and lifestyle effects on the suggested AMD metabolic fingerprints. We also observed a number of small metabolite variations potentially differentiating controls from early AMD. This is particularly relevant as these might represent specific signals to distinguish disease from non-disease status.

As discussed, MS has a much higher sensitivity than NMR, enabling the measurement of a broader range of metabolites. Therefore, we continued our investigations using MS metabolomics. In the Boston cohort (Laíns et al., 2017b), we observed that after controlling for age, gender, BMI and smoking status, 87 metabolites differed significantly between AMD cases and controls. Indeed, a summary score based on these 87 metabolites increased the ability to predict AMD cases, relative to clinical covariates alone. Of these metabolites, over half (48 metabolites) also differed significantly across AMD severity stages. Similar to what was described by Osborn et al. (2013), and what we observed using NMR, we identified significantly increased levels of dipeptides and amino acids metabolites in patients with AMD, including a significant role for alanine and aspartate metabolism. However, the majority of the identified significant metabolites were involved in lipid metabolism, in particular glycerophospholipid metabolism. Glycerophospholipids are a major component of cell membranes and are especially enriched in neural membranes. Indeed, changes in glycerophospholipids and their metabolism have been extensively investigated in neurodegeneration and in several chronic neurological diseases, (Farooqui et al., 2000; Kosicek and Hecimovic, 2013) and metabolomics is currently considered a promising tool to identify valid biomarkers and new targets in Alzheimer disease (Mapstone et al., 2017; Proitsi et al., 2017; Whiley et al., 2014). This is relevant because AMD is a neurodegenerative disease of the retina and shares important features and pathologic mechanisms with Alzheimer disease (Ermilov and Nesterova, 2016; Kaarniranta et al., 2011; Ohno-Matsui, 2011; Sivak, 2013). In Alzheimer disease, glycerophospholipids have been shown to be reduced, and to play a central role in its pathogenesis (Farooqui, 2012; Frisardi et al., 2011). Using MS metabolomics and lipidomics, Mapstone et al. (2014) identified a panel of ten plasma lipids, most of them phosphatidylcholines (a class of glycerophospholipids), that predicted with high accuracy conversion to mild cognitive impairment and to frank Alzheimer disease in elderly subjects.

Interestingly, Li et al. (2016) also described a significant role of glycerophospholipids' plasma metabolites in patients with polypoidal choroidal vasculopathy (PCV) (a subtype of wet AMD), as compared to controls (n = 21 and 19, respectively). Importantly, the authors focused only on lipid metabolites (lipidomics), assessed with untargeted MS. Their results revealed that the glycerophospholipid pathway was one of the most significant pathways involved in the separation of their study groups. However, the key indicator seemed to be platelet activating factor (PAF), which was significantly higher in patients with PCV. As the authors pointed out, PAF is an endogenous bioactive phospholipid that plays an important role in angiogenesis promotion.

Metabolomics has also been used to increase the current understanding of AMD pathophysiology at a cellular level, namely related to the formation of drusen and other sub-RPE deposits. These are the clinical and pathological hallmark of AMD, and their features are known to be linked to AMD progression. Thomson at al (Thompson et al., 2015). used a combination of analytical techniques including SIMS imaging (one of the techniques for MSI) to study the composition and origin of focal (drusen) or diffuse (basal linear and basal laminar) sub-RPE deposits. The authors isolated sub-RPE deposits from human cadaveric eyes and described that they contained hydroxyapatite spherules with cholesterol-cores, which provided a scaffold for proteins to adhere. Based on this work, they postulated that the process of drusen and basal deposits formation starts with the deposition of insoluble hydroxyapatite spherules around naturally occurring, extracellular lipid droplets that contain cholesterol at the RPE/choroid interface. Then, proteins and lipids attach to these shells, initiating or supporting the growth of these sub-RPE deposits. This is in agreement with important work by Dr. Curcio's lab (Pilgrim et al., 2017) that developed a RPE cell culture model able to produce sub-RPE deposits containing hydroxyapatite and lipids, among other elements, without outer segment supplementation.

5.4.1. Animal model investigations

Using an aged-mouse model, Rowan et al. (2017) found further evidence of the importance of lipid metabolites on AMD. The authors performed an integrative assessment of the metabolome (urine and plasma) with the microbiome, to better understand the effect of high-

glycemic (HG) *versus* low-glycemic (LG) diets on AMD features, such as photoreceptor cell damage and RPE abnormalities. Plasma metabolomics was assessed by MS and urine metabolomics by NMR. The authors described the identification of 26 metabolites common to both fluids, although the results should be evaluated with the caveat that the two fluids were assessed using two different techniques. The comparison of the plasma and urinary metabolomic profile of mice fed with HG, LG, and switched from HG to LG diets revealed significant differences. Mice with HG diets presented higher levels of lipids and higher retinal damage scores, while mice from the LG diet group presented higher levels of carbohydrates and amino acids, as well as lower retinal damage scores. Additionally, the authors tried to identify metabolites that could be associated with the presence of AMD features, verifying that eight of them, including a phosphatidylcholine and carnitine, enabled a good separation (high area under the curve) between mice with a high retinal damage score and non-affected animals. In the microbiome, an association was observed with metabolites modulated by the abundance of microbiota, such as serotonin, whose higher levels were associated with less retinal damage. The microbiome composition was different among the three different dietary groups (HG, LG and switch from HG to LG), but interestingly in the mice that were switched from HG to LG, the microbiome was restored to the LG mice. The microbiome of HG mice was enriched in Clostridia and Firmicutes, and both of these were related to a more advanced retinal damage score. These findings led the authors to emphasize the role of diet on AMD pathogenesis. Finally, using analytical techniques to integrate all data, Rowan et al. (2017) assessed diet-metabolome-AMD features interactions. These seemed to be mostly driven by a central hub including three lipids, serotonin and lysophosphatidylethanolamine.

5.5. Macular telangiectasia

Macular telangiectasia type 2 (MacTel) is a rare neurodegenerative disease specifically affecting the macular region of the retina, causing progressive central vision loss – Fig. 6. It has a late onset, typically presenting between 40 and 60 years of age (Charbel Issa et al., 2013). While there is strong evidence for genetic etiology, no causative gene or genes have been identified.

While the clinical manifestations of the disease are primarily restricted to the macular region of the retina, there have been reported associations between MacTel and systemic abnormalities including diabetes, obesity, hypertension and increased BMI (Charbel Issa et al., 2013). Metabolomic MS analysis was run by the MacTel consortium on patient serum in the hope of identifying systemic metabolite changes that associate with the disease (Charbel Issa et al., 2013). Fasting serum was collected for 50 MacTel patients and 50 control subjects (matched for age, ethnicity and diabetes). Interestingly, plasma metabolite differences were detected between the MacTel and control cohort. In particular three amino acids - serine, glycine and threonine - were significantly decreased in plasma from MacTel patients. Concurrently, a genome-wide association analysis of MacTel subjects identified disease-associated genetic loci that link to serine and glycine metabolism. This study highlights the application of patient-derived samples, like serum/plasma and possibly cells, to investigate disease-specific questions, even when the disease is rare and samples are not derived directly from the diseased tissue.

5.6. Others

Young et al. (2009) performed one of the first studies applying metabolomics to the study of eye diseases. The authors obtained vitreous humor samples from 42 patients undergoing PPV for several vitreoretinal disorders, thus including a very heterogeneous cohort. Most of the patients had a diagnosis of chronic non-infectious uveitis (n = 20, including panuveitis, pars planitis and Fuch's heterochromic cyclitis) or lens induced uveitis (n = 9). These two groups presented a distinct vitreous metabolome as assessed by three distinct analytical methods. The authors performed a limited assessment of the metabolites responsible for this separation, but these included glucose, oxaloacetate and urea, which were increased in samples from eyes with lens induced uveitis. This is an acute condition, thus being logical to see increased levels of inflammatory metabolites (such as oxaloacetate and urea) as compared to a more chronic process. Importantly, most of these patients were receiving steroids for treatment, which differed between the two groups (lens induced uveitis with topical steroids, while chronic uveitis with oral steroids). This might have introduced an important bias. The authors also did not account for other potential confounding factors.

5.6.1. Animal model investigations

Loss or reduction of blood flow and/or induction of hypoxia in the retina are thought to be causative or confounding factors in many retinal disorders, including age-related macular degeneration, diabetic retinopathy and glaucoma (Narayanan et al., 2013; Kurihara et al., 2016; Osborne et al., 2004). Therefore, animal models of reduced blood flow or retinal hypoxia coupled with metabolomics tools have been used to provide insights into the metabolic signatures that might associate with these retinal diseases.

D'Alessandro et al. (2014) used an *ex vivo* mouse model of retinal ischemia to study the consequences of reduced perfusion to the retina, as well as to test the capacity of different peptide therapeutics to recover some of the damage. In their model, the retina was removed and ischemia was induced *ex vivo* by putting the tissue in an air-tight container and adding sodium azide to block mitochondrial oxygen

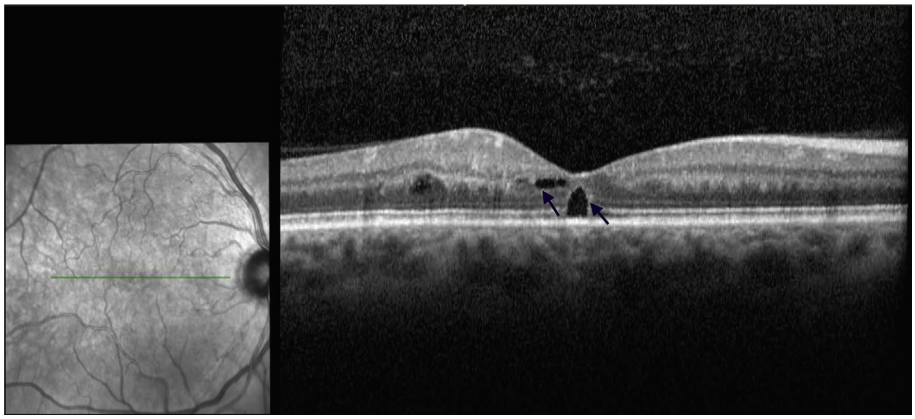

Fig. 6. Infra-red and optical coherence tomography of a right eye with macular telangiectasia. The image demonstrates abnormal vessels in the perifoveal area and hyporeflective spaces in the inner and outer retina (blue arrows).

consumption. Untargeted metabolic profiling was used to look at the changes that occur from these treatments compared to controls, as well as the ability of candidate peptides treatment to recover the effects. Metabolite changes of interest arising for the untargeted approach were validated by multiple reaction monitoring (MRM). The *ex vivo* ischemia model leads to extensive cell apoptosis and, not surprisingly, a rapid alteration in the levels of numerous metabolites. Notably, they found metabolites related to oxidative stress, including glutathione, peroxidized lipids and nitric oxide metabolites were all increased due to ischemia with varying levels or rescue by the peptides. Central carbon metabolism was altered with an accumulation of lactate, decreases in glycolytic intermediates and elevation metabolites of the pentose phosphate pathway. They also observed altered levels of purines metabolites, something seen also in the oxygen-induced retinopathy mouse model (OIR) (Paris et al., 2016).

It is well established that ischemia and the total loss of blood supply to the retina results in metabolic dysfunction and photoreceptor death. Interestingly, recent work by Kurihara et al. (2016) has shown that induction of hypoxia signaling alone specifically in the RPE is sufficient to cause substantial photoreceptor dysfunction and death, mirroring features of AMD. The authors used high-resolution untargeted metabolomics to verify a lipid dysregulation in a mouse model of RPE hypoxia signaling. They observed that numerous acylcarnitines were increased in the mouse RPE/choroid complex and this phenotype could be corrected with the deletion of the HIF genes and the prevention of hypoxia signaling (Kurihara et al., 2016).

Hibernating animals experience dramatically reduced blood flow to their retinas during hibernation, essentially a non-pathological form of ischemia. Metabolic adaptations are used to deal with the reduced blood flow and result in increased resistance to ischemia. Luan et al. (2018) studied the retinas of hibernating ground squirrels by coupling transcriptome analysis with metabolomics to reveal the metabolic pathways that are altered in the hibernation state. Winter-awake animals were compared to deep torpor (hibernating) animals (6 per group) and untargeted analysis was performed using both GC/MS and LC/MS platforms. The metabolomics data was integrated with transcriptome data and used in pathway analysis. Analysis revealed a shift from carbohydrate metabolism to lipid oxidation, similar to other tissues and what is expected in a hibernating or fasting state. However, some amino acid levels (including acetyled amino acids) were increased, which appears unique to hibernating retinal metabolism and different than other hibernating tissues (Luan et al., 2018; Nelson et al., 2009). Understanding the metabolic adaptations that allow the retina to withstand low blood flow may be informative to treating retina diseases connected to ischemia and/or hypoxia.

To find a potential biomarker for postnatal hypoxia induced brain injury Solberg et al. (2013) used a porcine model of hypoxia and examined the retinas as representative neuronal tissue. Five piglets were used in a control and treatment group, and 8 metabolites were identified that surpassed thorough statistical analysis. CDP-choline was identified and further validated and quantified with targeted mass spectrometry as significantly increased in the hypoxic retinas. Further work will be needed to determine if CDP-choline has value as a biomarker, including testing the levels in plasma.

5.7. Limitations

The application of metabolomics to the study of retinal diseases, and the studies that have been published to date, have important limitations. These include both limitations specific to the different study designs, and limitations of metabolomics itself.

Of the studies mentioned in this manuscript, only a few (Tan et al., 2016a,b; Yu et al., 2015; Li et al., 2014; Osborn et al., 2013; Laíns et al., 2017b) reported un-annotated metabolites (i.e. with no identification available on databases such as HMDB). Un-annotated metabolites are important to describe, because if they are associated with disease or with health, their omission can introduce bias into the reported results. Therefore, they should be described and taken into account in all metabolomic studies. Un-annotated metabolites remain an important problem in metabolomics, since to date no complete characterization of any organismal metabolome has been reported, dramatically contrasting with the genome (Viant et al., 2017). This is due to several issues, including: the vast heterogeneity of metabolites; their wide variation in concentration and size (for example, of approximately 4000 metabolites in human serum, concentrations range over 11 orders of magnitude with the most abundant metabolites measured at millimolar concentrations, and low abundance metabolites measured at picomolar concentrations (Psychogios et al., 2011); and their large number and distinct psychochemical properties (for example, differences in polarity among groups or families of metabolites demand different extraction procedures). Because of the higher sensitivity of MS, this is particularly evident in studies using this analytical approach (Petras et al., 2017). Un-annotated metabolites have been the focus of recent attention, and an international task group is in place to help coordinate metabolome annotation (Viant et al., 2017). Currently, among the most commonly used public available metabolomic databases are the Human Metabolome Database (http://www.hmdb.ca/) and METLIN (http://metlin.scripps.edu/), and these are certainly crucial tools to address the challenge of metabolomic annotation (Kell and Oliver, 2016).

Most studies included in this manuscript provide a detailed description of their methods for sample collection and processing (Kohler et al., 2016), however, several studies lack quality control samples. This is particularly relevant when large series of samples are analyzed, to control for the analytical systems performance and identify eventual drifts (Scalbert et al., 2009). Quality control samples are commonly obtained by sample pooling (a pooled sample to represent all the samples to be analyzed) (Barnes et al., 2016a), as performed for example by Li et al. (2014), Yu et al. (2015) and Chen et al. (2016) or can be achieved by using internal standards from different laboratories (Hopiavuori et al., 2017). In any case, a laboratory should analyze and report the variation that occurs for repeated analysis of the same sample, for multiple extractions of the same sample on one day, and for multiple extractions of the same sample over a period of time (Barnes et al., 2016a). Before data analysis, data quality control procedures should also take place, namely to identify and assess the number of metabolites with missing data or undetected levels, to identify outliers, and to analyze the normality of the data (Kumar et al., 2017). Several techniques are available to deal with these distinct data analysis challenges, and it is crucial to report them (Di Guida et al., 2016). In the field of metabolomics applied to retinal diseases few papers (Sun et al., 2014; Tan et al., 2016a,b; Laíns et al., 2017b; Chen et al., 2016) presented their approach to deal with these aspects.

In terms of data analysis for metabolomics studies, as described in the respective section of this manuscript, it is also important to account for the fact that multiple hypotheses are tested (Hopiavuori et al., 2017; Tan et al., 2016a,b; Li et al., 2014; Yu et al., 2015; Osborn et al., 2013; Laíns et al., 2017a; de la Barca et al., 2017; Rowan et al., 2017; Chen et al., 2016). Another important concept relates to the requirement of validating metabolomics results, especially if they refer to the identification of potential biomarkers. The ideal approach is to assess an independent cohort (Monteiro et al., 2013), which so far, to our knowledge, has only been partially attempted by one of the studies published in the field of vitreoretinal diseases(Chen et al., 2016). Also, few studies (Li et al., 2016), (Chen et al., 2016), (Laíns et al., 2017b), (Rowan et al., 2017) assessed the performance of their potential metabolomic biomarkers using ROC analysis, which, as mentioned, is the standard method for assessment of biomarkers performance; and few groups commented on other approaches to validate their results, namely concerning PLS-DA analysis (Li et al., 2014; Yu et al., 2015; Osborn et al., 2013; Laíns et al., 2017a; Locci et al., 2014; Young et al., 2009). Importantly, these are mostly cross-sectional association studies, so no causality can be inferred. The observed changes in the metabolomic

profiles might be related to causal mechanisms and may also represent the downstream effect of the metabolomic derangements in the distinct vitreoretinal diseases. To understand the causal roles of metabolites, one should ideally develop prospective longitudinal studies that allow for future disease risks to be evaluated on the basis of both genetic and metabolic information. This was performed by Rowan et al. (2017), using an animal model to the study of AMD. These authors were also the only group who assessed the influence of diet in the evaluated metabolomic profiles. In the remaining studies, no assessments have been performed in terms of the potential influence that dietary patterns and genetic profiles may have in the described metabolomic profiles. As stated, all these factors contribute to the overall metabolomic profile, and can obscure or enhance differences, thus representing a potential source of bias (Monteiro et al., 2013).

Despite its relevance for the understanding of disease biology and mechanisms, a comprehensive integration of metabolomic profiles among different biospecimens (i.e. urine, plasma and feces) has also only been attempted in animal models (Rowan et al., 2017; Tan et al., 2016a,b). This is important because, as mentioned, metabolomic profiles vary depending on the biofluid assessed and very little literature is available correlating these data. For example, it would be interesting to understand if and how the described metabolomic findings on plasma samples of diabetic retinopathy patients (Barba et al., 2010; Paris et al., 2016) relate to vitreous profiles (Chen et al., 2016), as at a first glance they seem to be mostly unrelated. In all these analyses, however, one needs to bear in mind that the metabolomic characterization reflects the nature of the biospecimen assessed, i.e. for example, plasma reflecting the whole organism profile, while urine is a filtration of the plasma, and vitreous or retinal tissue reflect the local eye environment. Indeed, it is important to further emphasize that, as pointed by Tan et al.(2016a,b), despite its proximity to the retina, the vitreous does not necessarily mirror retina metabolomic changes. Funding limitations, study design constraints, and the complexity of the statistical approaches required to perform association studies across biospecimens are likely the cause of the current lack of data in this field, particularly for human subjects. However, methods for robust data analysis have been developed, (Do et al., 2017; Do et al., 2015; Feng et al., 2016; Xia and Wishart, 2010) and can unravel unparalleled data for the understanding of disease mechanisms, and also to assess the biological relevance of peripheral (i.e. in the blood and urine) biofluid biomarkers. Pathway analysis methods have also been applied to a very limited extent in the field of vitreoretinal conditions, (Laíns et al., 2017b; Chen et al., 2016; Li et al., 2016; Yu et al., 2015) and they are crucial to meet these opportunities provided by metabolomics to increase the current understanding of disease pathophysiology (as described in the corresponding section). While in this manuscript it would had been ideal to organize published data by metabolic pathway and physiologic system, published studies do not provide sufficient information to permit this. Indeed, this is an opportunity for future work.

The inclusion of insufficient sample sizes is also a very important limitation and is problematic in the application of metabolomics across most medical fields. As described, there is currently no standard method for the determination of sample size when designing a metabolomic experiment (Blaise et al., 2016; Nyamundanda et al., 2013), but several tools are available for its estimation, and minimum numbers for animal and human studies have been reported (Barnes et al., 2016a). Our group has published on AMD human plasma metabolomics following these recommendations ($n \geq 30$ for each study group), (Laíns et al., 2017a; Laíns et al., 2017b) but this is not the case for most of the remaining literature. Indeed, published studies with animal tissue included sample sizes varying between one (Hopiavuori et al., 2017) to seven (Tan et al., 2016a,b) samples per group; and studies with human vitreous samples included as low as eight samples per group (Li et al., 2014). For both animal and human studies, metabolomic studies have been limited by the difficulty in obtaining adequate healthy control groups, matched for relevant potential confounding factors, such as age or gender. Collaborative studies, including several institutions from the same country or different countries, are probably the best strategy to overcome this problem, especially for human clinical studies.

In summary, although the potential of the metabolic phenotyping strategy appears obvious, so far, its application to the field of retinal diseases is limited by a lack of well-designed and appropriately powered studies, as well as the lack of validation cohorts (Holmes et al., 2015). The clinical applicability of metabolomics strongly relies on the quality and accuracy of the acquired data. Study design should be planned in advance, including all crucial steps to ensure the highest data quality and lowest analytical variability. This includes subject/sample selection, sample collection, sample size, sample handling and storage conditions, preparation and data analysis. Also, new methods should always be evaluated in comparison with the current clinical standards, to ensure that they represent an actual benefit.

5.8. Future directions

In the last few decades, the field of metabolomics has experienced an exponential growth, and holds great promise to significantly improve diagnosis, provide prognostic information and identify additional therapeutic targets. The potential of this approach for translation to the clinical environment is great. However, as described in this manuscript, the application of metabolomics to the study of vitreoretinal diseases is very limited, and not comparable to what has happened in other medical fields (Kohler et al., 2016). Clearly, we are still very far from clinical translation. Most studies were hindered by small sample sizes and data analysis constrains. However, we believe that there is a great opportunity for a wider use of metabolomics to further understand the mechanisms of several vitreoretinal diseases, as well as for developing diagnostic and prognostic biomarkers. Indeed, for certain conditions studied, such as AMD, the available results are encouraging, considering the reported biologically plausible metabolites and metabolomic pathways.

As summarized, most work performed so far in the field of vitreoretinal diseases relates to the identification of potential biomarkers. In this manuscript, we have pointed opportunities, current limitations and especially approaches to improve the application of metabolomics to that end. Indeed, we further highlight that there is a great need to developing highly predictive and robust biomarkers able to reflect the complex nature of diseases. In this section, however, we focus on other future directions not yet widely explored, which we believe can also aid clinical care and improve patients' outcomes.

One of the new concepts of modern medicine is "precision medicine" or "personalized medicine". According to the Precision Medicine Initiative, it takes into account individual variability in genes, environment and lifestyle for treatment and prevention. This means that differences between individuals are considered, instead of "one-size fits all approach". The goal is that ultimately clinicians can prescribe the right medicine to the right patient at the right time, with maximum efficacy and minimal toxicity, as well as predicting the susceptibility to disease onset among populations (Sun and Hu, 2016). Metabolomics is one of the most widely applicable areas for the development of precision medicine, as it mirrors genes, environment and lifestyle. For example, metabolomics can help in identifying disease subtypes and stratify patients with certain conditions. This is good to maximize patient benefit and minimize patient harm, and would be useful for example for AMD and DR, both complex diseases with a spectrum of phenotypes and pathways of progression. In clinical research, stratification can also enhance selection of appropriate trial participants, and thus increase their power and enhance the analysis of outcomes.

Another example, which has also not been explored in vitreoretinal diseases, is pharmacometabolomics/pharmacometabolomics, i.e. metabolomics applied to drug discovery and development. Metabolomics is expected to have the ability to identify markers of drug toxicity and efficacy that can accelerate drug discovery and assist to delineate

appropriate clinical plans (Cuperlovic-Culf and Culf, 2016; Tolstikov, 2016). For example, pharmacometabolomics has been used to assess toxicity induced by acetaminophen (Winnike et al., 2010), as well as to predict efficacy and adverse events of other drugs, such as oral anti-diabetics or statins (Sun and Hu, 2016; Wishart, 2016; Zhang et al., 2015). This is particularly important considering the global aging of the population. Typically, older patients have more comorbidities and polypharmacy, thus representing a bigger challenge due to the unknown interactions between drugs. Pharmacometabolomics can potentially provide a gateway to stratified medicine, by acting as a screening tool for selecting individuals according to their suitability for treatment with particular drugs (Nicholson et al., 2012a). This could be an interesting approach, for example, to study the efficacy of vitamin supplements to halt AMD progression; or the differences in response to anti-angiogenic treatments, namely why some patients do not respond as opposed to others, and the differences among the different agents. Due to the current costs of these medications, and an increasing awareness on the great need for cost-effectiveness in patient care, this is of outmost importance. Metabolomics is used to probe the real-world nature of biochemical functionality and is sensitive to both gene and environmental influences; therefore, it is likely to be more practical than gene-based measurements of response to therapy. Indeed, the concept of metabolomics trajectory has been suggested (Nicholson et al., 2012b), and includes obtaining biofluid samples prior to, during, and after a patient undergoes treatment. The obtained information can be used to monitor effects of any treatment, and to predict outcomes. This will enable better outcomes instead of trial and error treatment regimes. Also, personalized medicine could be better achieved if instead of biosamples, wearable technologies could be developed and applied. An excellent example are glucose monitoring contact lenses, which are able to transfer biomarker discovery and assessment into an individual level (Badugu et al., 2018; Elsherif et al., 2018).

One can also speculate that there will be great power in combining data from multiple ‘omics, (Inouye et al., 2010; Sun and Hu, 2016) which can lead to greater insight and better characterization of complex diseases. To truly understand disease mechanisms, we need to adopt a systems biology approach combining multiple bio-organizational layers, such as the genome, proteome and metabolome (Holmes et al., 2015). Indeed, with the currently available platforms and options for data analysis, it is possible to analyze wide-ranging metabolomic phenotypes in association with genetic variance, disease-relevant phenotypes and lifestyle and environmental parameters, allowing dissection of the relative influences of these factors (Suhre and Gieger, 2012). This strategy can allow the visualization of a biological system on a global level. Namely, the so-called metabolome-genome wide association (GWAS) studies are becoming increasingly popular (Wang et al., 2017). The conventional GWAS studies focus on the association between single nucleotide polymorphisms (SNPs) and disease phenotypes (for example, disease *versus* non-disease). These require large sample sizes and often do not provide any information on the underlying biological processes. Conversely, metabolome-GWAS studies are more highly powered and, by using metabolomic profiles as intermediate traits, they provide information on the biology of the disease association. This has been demonstrated in several medical fields, including cancer (Wang et al., 2017), and can be of great utility for vitreoretinal conditions that lack a complete understanding of their pathophysiology. If novel pathways and their interactions are unraveled, this can lead to the identification of novel targets and the development of therapies directed to them. This is crucial considering the current lack of treatment for conditions like macular telangiectasia, and for the dry and atrophic forms of AMD, among others. For AMD, there is also still a great need to understand the mechanisms involved in progression, and to develop strategies to halt it, and thus reduce the burden of this blinding condition. Of note, ‘omics integrative analyses remain in their early stages and clinical utility has yet to be demonstrated. Machine-learning approaches can be a powerful tool to support these and other metabolomics data analysis (Zhou et al., 2017). Indeed, improvements in technology and computational solutions are still required, and ideally one should be able to link metabolomic with dynamic metadata from patients to predict risks and prognosis (Trivedi et al., 2017).

In the future, imaging metabolomics (as described, enabling the study of the distribution of metabolites within a tissue) will also likely play an increasingly important role to provide new insights into the biological processes at systems biology level (Sun et al., 2014). The retina is a highly specialized tissue, with a complex anatomy. Each layer has a different morphology and function. The ability to assess and obtain qualitative and quantitative metabolomics data without disturbing this complex anatomy in its native environment is extremely valuable. It can facilitate the understanding of disease processes, and the identification of novel biomarkers. This is particularly true if one can integrate these data with the metabolomic phenotype of easily accessible biofluids, bearing in mind that these represent distinct biological environments, thus the detected biomarkers might differ.

Importantly, to make the most out of metabolomics potential and open new avenues in research and patient care, it is crucial to establish multidisciplinary teams, where clinicians, biostatisticians, bioinformatics, geneticists, and others, work synergistically and capitalize their knowledge in the different required fields. Cross-disciplinary efforts, as well as translational collaborations between academic institutions, pharmaceutical agencies and diagnostic companies are crucial to move forward (Kohler et al., 2016).

In conclusion, at present metabolomics is research laboratory-based, and needs to become more practical to be easily implemented in the clinic (Trivedi et al., 2017). This represents a great challenge for the field of metabolomics in general, and much work lies ahead. For this to happen, one of the most crucial requirements is to standardize and harmonize metabolomic phenotyping and biomarker modelling procedures, and to improve the current publicly available databases and promote data sharing (Holmes et al., 2015).

We envision that metabolomics, and its integration with other ‘omics approaches, will be increasingly applied in the coming years to the study of vitreoretinal diseases. This will lead to increased knowledge on their mechanisms and pathophysiology, the identification of diagnostic and risk-prediction biomarkers, and the assessment of efficacy and toxicity of several drugs. By contributing for better diagnostics, better patient selection, better treatment and cost-effectiveness of treatment strategies, it will improve patient outcomes and offer higher-precision medicine of high value. We live in the era of precision medicine, and we are now in a position to develop global systems-biology care for conditions that are characterized by gene–environment interactions. The field of vitreoretinal diseases will certainly benefit from this approach. In this manuscript, we provide tools for a better understanding of metabolomics and its potential in clinical translational research and management of retinal diseases.

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References

D'Alessandro, A., Cervia, D., Catalani, E., Gevi, F., Zolla, L., Casini, G., 2014. Protective effects of the neuropeptides PACAP, substance P and the somatostatin analogue octreotide in retinal ischemia: a metabolomic analysis. Mol. Biosyst. 10, 1290. https://doi.org/10.1039/c3mb70362b.

D'Amico, D.J., 2008. Primary retinal detachment. N. Engl. J. Med. 359, 2346–2354. https://doi.org/10.1056/NEJMcp0804591.

Abcouwer, S.F., Gardner, T.W., 2014. Diabetic retinopathy: loss of neuroretinal adaptation to the diabetic metabolic environment. Ann. N. Y. Acad. Sci. 1311, 174–190. https://doi.org/10.1111/nyas.12412.

Acharjee, A., Finkers, R., Visser, R.G., Maliepaard, C., 2012. Comparison of regularized regression methods for ~Omics data. J. Postgenomics Drug Biomark. Dev. 03. https://doi.org/10.4172/2153-0769.1000126.

Alonso, A., Marsal, S., Julià, A., 2015. Analytical methods in untargeted metabolomics: state of the art in 2015. Front. Bioeng. Biotechnol. 3, 23. https://doi.org/10.3389/fbioe.2015.00023.

Álvarez-Sánchez, B., Priego-Capote, F., Luque de Castro, M.D., 2012. Study of sample preparation for metabolomic profiling of human saliva by liquid chromatography-time of flight/mass spectrometry. J. Chromatogr. A 1248, 178–181. https://doi.org/10.1016/j.chroma.2012.05.029.

Ammerlaan, W., Trezzi, J.-P., Lescuyer, P., Mathay, C., Hiller, K., Betsou, F., 2014. Method validation for preparing serum and plasma samples from human blood for downstream proteomic, metabolomic, and circulating nucleic acid-based applications. Biopreserv. Biobanking 12, 269–280. https://doi.org/10.1089/bio.2014.0003.

Assel, M.J., Li, F., Wang, Y., Allen, A.S., Baggerly, K.A., Vickers, A.J., 2018. Genetic polymorphisms of CFH and ARMS2 do not predict response to antioxidants and zinc in patients with age-related macular degeneration. Ophthalmology 125, 391–397. https://doi.org/10.1016/j.ophtha.2017.09.008.

Badugu, R., Reece, E.A., Lakowicz, J.R., 2018. Glucose-sensitive silicone hydrogel contact lens toward tear glucose monitoring. J. Biomed. Optic. 23, 1. https://doi.org/10.1117/1.JBO.23.5.057005.

Barba, I., Garcia-Ramírez, M., Hernández, C., Alonso, M.A., Masmiquel, L., García-Dorado, D., Simó, R., 2010. Metabolic fingerprints of proliferative diabetic retinopathy: an ^{1}H-NMR–Based metabonomic approach using vitreous. Humor. Investig. Opthalmology Vis. Sci. 51, 4416. https://doi.org/10.1167/iovs.10-5348.

Barnes, S., Benton, H.P., Casazza, K., Cooper, S.J., Cui, X., Du, X., Engler, J., Kabarowski, J.H., Li, S., Pathmasiri, W., Prasain, J.K., Renfrow, M.B., Tiwari, H.K., 2016a. Training in metabolomics research. I. Designing the experiment, collecting and extracting samples and generating metabolomics data. J. Mass Spectrom. 51, 461–475. https://doi.org/10.1002/jms.3782.

Barnes, S., Benton, H.P., Casazza, K., Cooper, S.J., Cui, X., Du, X., Engler, J., Kabarowski, J.H., Li, S., Pathmasiri, W., Prasain, J.K., Renfrow, M.B., Tiwari, H.K., 2016b. Training in metabolomics research. II. Processing and statistical analysis of metabolomics data, metabolite identification, pathway analysis, applications of metabolomics and its future. J. Mass Spectrom. 51, 535–548. https://doi.org/10.1002/jms.3780.

Billoir, E., Navratil, V., Blaise, B.J., 2015. Sample size calculation in metabolic phenotyping studies. Briefings Bioinf. 16, 813–819. https://doi.org/10.1093/bib/bbu052.

Blaise, B.J., Navratil, V., Domange, C., Shintu, L., Dumas, M.-E., Elena-Herrmann, B., Emsley, L., Toulhoat, P., 2010. Two-dimensional statistical recoupling for the identification of perturbed metabolic networks from NMR spectroscopy. J. Proteome Res. 9, 4513–4520. https://doi.org/10.1021/pr1002615.

Blaise, B.J., Correia, G., Tin, A., Young, J.H., Vergnaud, A.-C., Lewis, M., Pearce, J.T.M., Elliott, P., Nicholson, J.K., Holmes, E., Ebbels, T.M.D., 2016. Power analysis and sample size determination in metabolic phenotyping. Anal. Chem. 88, 5179–5188. https://doi.org/10.1021/acs.analchem.6b00188.

Boulagnon, C., Garnotel, R., Fornes, P., Gillery, P., 2011. Post-mortem biochemistry of vitreous humor and glucose metabolism: an update. Clin. Chem. Lab. Med. 49, 1265–1270. https://doi.org/10.1515/CCLM.2011.638.

Bowrey, H.E., Anderson, D.M., Pallitto, P., Gutierrez, D.B., Fan, J., Crouch, R.K., Schey, K.L., Ablonczy, Z., 2016. Imaging mass spectrometry of the visual system: advancing the molecular understanding of retina degenerations. PROTEOMICS - Clin. Appl. 10, 391–402. https://doi.org/10.1002/prca.201500103.

Buck, A., Balluff, B., Voss, A., Langer, R., Zitzelsberger, H., Aichler, M., Walch, A., 2016. How suitable is matrix-assisted laser desorption/ionization-time-of-flight for metabolite imaging from clinical formalin-fixed and paraffin-embedded tissue samples in comparison to matrix-assisted laser desorption/ionization-fourier transform ion cyclotron resonance mass spectrometry? Anal. Chem. 88, 5281–5289. https://doi.org/10.1021/acs.analchem.6b00460.

Bujak, R., Daghir-Wojtkowiak, E., Kaliszan, R., Markuszewski, M.J., 2016. PLS-based and regularization-based methods for the selection of relevant variables in non-targeted metabolomics data. Front. Mol. Biosci. 3, 35. https://doi.org/10.3389/fmolb.2016.00035.

Cacciatore, S., Zadra, G., Bango, C., Penney, K.L., Tyekucheva, S., Yanes, O., Loda, M., 2017. Metabolic profiling in formalin-fixed and paraffin-embedded prostate cancer tissues. Mol. Canc. Res. 15, 439–447. https://doi.org/10.1158/1541-7786.MCR-16-0262.

Chan, E.C.Y., Pasikanti, K.K., Nicholson, J.K., 2011. Global urinary metabolic profiling procedures using gas chromatography?mass spectrometry. Nat. Protoc. 6, 1483–1499. https://doi.org/10.1038/nprot.2011.375.

Charbel Issa, P., Gillies, M.C., Chew, E.Y., Bird, A.C., Heeren, T.F.C., Peto, T., Holz, F.G., Scholl, H.P.N., 2013. Macular telangiectasia type 2. Prog. Retin. Eye Res. 34, 49–77. https://doi.org/10.1016/J.PRETEYERES.2012.11.002.

Chen, L., Zhou, L., Chan, E.C.Y., Neo, J., Beuerman, R.W., 2011. Characterization of the human tear metabolome by LC-MS/MS. J. Proteome Res. 10, 4876–4882. https://doi.org/10.1021/pr2004874.

Chen, L., Cheng, C.-Y., Choi, H., Ikram, M.K., Sabanayagam, C., Tan, G.S.W., Tian, D., Zhang, L., Venkatesan, G., Tai, E.S., Wang, J.J., Mitchell, P., Cheung, C.M.G., Beuerman, R.W., Zhou, L., Chan, E.C.Y., Wong, T.Y., 2016. Plasma metabonomic profiling of diabetic retinopathy. Diabetes 65, 1099–1108. https://doi.org/10.2337/db15-0661.

Chetwynd, A.J., Dunn, W.B., Rodriguez-Blanco, G., 2017. Collection and preparation of clinical samples for metabolomics. In: Advances in Experimental Medicine and Biology, pp. 19–44. https://doi.org/10.1007/978-3-319-47656-8_2.

Chi, Y.-Y., Gribbin, M.J., Johnson, J.L., Muller, K.E., 2014. Power calculation for overall hypothesis testing with high-dimensional commensurate outcomes. Stat. Med. 33, 812–827. https://doi.org/10.1002/sim.5986.

Chong, J., Soufan, O., Li, C., Caraus, I., Li, S., Bourque, G., Wishart, D.S., Xia, J., 2018. MetaboAnalyst 4.0: towards more transparent and integrative metabolomics analysis. Nucleic Acids Res. https://doi.org/10.1093/nar/gky310.

Ciprian, D., 2015. The pathogeny of proliferative vitreoretinopathy. Rom J Ophthalmol 59, 88–92.

Coman, C., Solari, F.A., Hentschel, A., Sickmann, A., Zahedi, R.P., Ahrends, R., 2016. Simultaneous metabolite, protein, lipid extraction (SIMPLEX): a combinatorial multimolecular omics approach for systems biology. Mol. Cell. Proteomics 15, 1453–1466. https://doi.org/10.1074/mcp.M115.053702.

Crick, F., 1970. Central dogma of molecular biology. Nature 227, 561–563.

Crutchfield, C.A., Thomas, S.N., Sokoll, L.J., Chan, D.W., 2016. Advances in mass spectrometry-based clinical biomarker discovery. Clin. Proteonomics 13, 1. https://doi.org/10.1186/s12014-015-9102-9.

Cuilla, T.A., Ying, G., Maguire, M.G., Martin, D.F., Jaffe, G.J., Grunwald, J.E., Daniel, E., Toth, C.A., Toth, C.A., Comparison of Age-Related Macular Degeneration Treatments Trials Research Group, 2015. Influence of the vitreomacular interface on treatment outcomes in the comparison of age-related macular degeneration treatments trials. Ophthalmology 122, 1203–1211. https://doi.org/10.1016/j.ophtha.2015.02.031.

Cuperlovic-Culf, M., 2018. Machine learning methods for analysis of metabolic data and metabolic pathway modeling. Metabolites 8, 4. https://doi.org/10.3390/metabo8010004.

Cuperlovic-Culf, M., Culf, A.S., 2016. Applied metabolomics in drug discovery. Expet Opin. Drug Discov. 11, 759–770. https://doi.org/10.1080/17460441.2016.1195365.

Dane, A.D., Hendriks, M.M.W.B., Reijmers, T.H., Harms, A.C., Troost, J., Vreeken, R.J., Boomsma, D.I., van Duijn, C.M., Slagboom, E.P., Hankemeier, T., 2014. Integrating metabolomics profiling measurements across multiple biobanks. Anal. Chem. 86, 4110–4114. https://doi.org/10.1021/ac404191a.

de la Barca, J.M.C., Huang, N.-T., Jiao, H., Tessier, L., Gadras, C., Simard, G., Natoli, R., Tcherkez, G., Reynier, P., Valter, K., 2017. Retinal metabolic events in preconditioning light stress as revealed by wide-spectrum targeted metabolomics. Metabolomics 13, 22. https://doi.org/10.1007/s11306-016-1156-9.

Di Guida, R., Engel, J., Allwood, J.W., Weber, R.J.M., Jones, M.R., Sommer, U., Viant, M.R., Dunn, W.B., 2016. Non-targeted UHPLC-MS metabolomic data processing methods: a comparative investigation of normalisation, missing value imputation, transformation and scaling. Metabolomics 12, 93. https://doi.org/10.1007/s11306-016-1030-9.

Diaz, S.O., Barros, A.S., Goodfellow, B.J., Duarte, I.F., Galhano, E., Pita, C., Almeida, M. do C., Carreira, I.M., Gil, A.M., 2013. Second trimester maternal urine for the diagnosis of trisomy 21 and prediction of poor pregnancy outcomes. J. Proteome Res. 12, 2946–2957. https://doi.org/10.1021/pr4002355.

Do, K.T., Kastenmüller, G., Mook-Kanamori, D.O., Yousri, N.A., Theis, F.J., Suhre, K., Krumsiek, J., 2015. Network-based approach for analyzing intra- and interfluid metabolite associations in human blood, urine, and saliva. J. Proteome Res. 14, 1183–1194. https://doi.org/10.1021/pr501130a.

Do, K.T., Pietzner, M., Rasp, D.J., Friedrich, N., Nauck, M., Kocher, T., Suhre, K., Mook-Kanamori, D.O., Kastenmüller, G., Krumsiek, J., 2017. Phenotype-driven identification of modules in a hierarchical map of multifluid metabolic correlations. npj Syst. Biol. Appl. 3, 28. https://doi.org/10.1038/s41540-017-0029-9.

Du, J., Rountree, A., Cleghorn, W.M., Contreras, L., Lindsay, K.J., Sadilek, M., Gu, H., Djukovic, D., Raftery, D., Satrústegui, J., Kanow, M., Chan, L., Tsang, S.H., Sweet, I.R., Hurley, J.B., 2016. Phototransduction influences metabolic flux and nucleotide metabolism in mouse retina. J. Biol. Chem. 291, 4698–4710. https://doi.org/10.1074/jbc.M115.698985.

Dunn, W.B., Broadhurst, D., Begley, P., Zelena, E., Francis-McIntyre, S., Anderson, N., Brown, M., Knowles, J.D., Halsall, A., Haselden, J.N., Nicholls, A.W., Wilson, I.D., Kell, D.B., Goodacre, R., 2011a. Procedures for large-scale metabolic profiling of serum and plasma using gas chromatography and liquid chromatography coupled to mass spectrometry. Nat. Protoc. 6, 1060–1083. https://doi.org/10.1038/nprot.2011.335.

Dunn, W.B., Broadhurst, D., Begley, P., Zelena, E., Francis-McIntyre, S., Anderson, N., Brown, M., Knowles, J.D., Halsall, A., Haselden, J.N., Nicholls, A.W., Wilson, I.D., Kell, D.B., Goodacre, R., Human Serum Metabolome (HUSERMET) Consortium, 2011b. Procedures for large-scale metabolic profiling of serum and plasma using gas chromatography and liquid chromatography coupled to mass spectrometry. Nat. Protoc. 6, 1060–1083. https://doi.org/10.1038/nprot.2011.335.

Elsherif, M., Hassan, M.U., Yetisen, A.K., Butt, H., 2018. Wearable contact lens biosensors for continuous glucose monitoring using smartphones. ACS Nano 12, 5452–5462. https://doi.org/10.1021/acsnano.8b00829.

Emwas, A.-H.M., 2015. The strengths and weaknesses of NMR spectroscopy and mass spectrometry with particular focus on metabolomics research. In: Methods in Molecular Biology. Clifton, N.J., pp. 161–193. https://doi.org/10.1007/978-1-4939-2377-9_13.

Emwas, A.-H., Roy, R., McKay, R.T., Ryan, D., Brennan, L., Tenori, L., Luchinat, C., Gao, X., Zeri, A.C., Gowda, G.A.N., Raftery, D., Steinbeck, C., Salek, R.M., Wishart, D.S., 2016. Recommendations and standardization of biomarker quantification using NMR-based metabolomics with particular focus on urinary analysis. J. Proteome Res. 15, 360–373. https://doi.org/10.1021/acs.jproteome.5b00885.

Ermilov, V.V., Nesterova, A.A., 2016. β-Amyloidopathy in the pathogenesis of age-related macular degeneration in correlation with neurodegenerative diseases. In: Advances in Experimental Medicine and Biology, pp. 119–125. https://doi.org/10.1007/978-3-319-17121-0_17.

Fang, M., Ivanisevic, J., Benton, H.P., Johnson, C.H., Patti, G.J., Hoang, L.T.,

Uritboonthai, W., Kurczy, M.E., Siuzdak, G., 2015. Thermal degradation of small molecules: a global metabolomic investigation. Anal. Chem. 87, 10935–10941. https://doi.org/10.1021/acs.analchem.5b03003.

Farooqui, A.A., 2012. Lipid mediators and their metabolism in the nucleous: implications for Alzheimer's disease. J. Alzheimers. Dis. 30 (Suppl. 2), S163–S178. https://doi.org/10.3233/JAD-2011-111085.

Farooqui, A.A., Horrocks, L.A., Farooqui, T., 2000. Glycerophospholipids in brain: their metabolism, incorporation into membranes, functions, and involvement in neurological disorders. Chem. Phys. Lipids 106, 1–29.

Federation of American Societies for Experimental Biology, C, Mean, R., Janssen, B.J., Hedlund, L.W., 2010. Federation proceedings., the FASEB journal. Federation of American Societies for Experimental Biology.

Feng, Q., Liu, Z., Zhong, S., Li, R., Xia, H., Jie, Z., Wen, B., Chen, X., Yan, W., Fan, Y., Guo, Z., Meng, N., Chen, J., Yu, X., Zhang, Z., Kristiansen, K., Wang, J., Xu, X., He, K., Li, G., 2016. Integrated metabolomics and metagenomics analysis of plasma and urine identified microbial metabolites associated with coronary heart disease. Sci. Rep. 6, 22525. https://doi.org/10.1038/srep22525.

Fiehn, O., 2002. Metabolomics–the link between genotypes and phenotypes. Plant Mol. Biol. 48, 155–171.

Frisardi, V., Panza, F., Seripa, D., Farooqui, T., Farooqui, A.A., 2011. Glycerophospholipids and glycerophospholipid-derived lipid mediators: a complex meshwork in Alzheimer's disease pathology. Prog. Lipid Res. 50, 313–330. https://doi.org/10.1016/j.plipres.2011.06.001.

Gessel, M.M., Norris, J.L., Caprioli, R.M., 2014. MALDI imaging mass spectrometry: spatial molecular analysis to enable a new age of discovery. J. Proteomics 107, 71–82. https://doi.org/10.1016/j.jprot.2014.03.021.

Ghosal, S., Nunley, A., Mahbod, P., Lewis, A.G., Smith, E.P., Tong, J., D'Alessio, D.A., Herman, J.P., 2015. Mouse handling limits the impact of stress on metabolic endpoints. Physiol. Behav. 150, 31–37. https://doi.org/10.1016/J.PHYSBEH.2015.06.021.

Gika, H.G., Theodoridis, G.A., Wilson, I.D., 2008. Liquid chromatography and ultra-performance liquid chromatography-mass spectrometry fingerprinting of human urine: sample stability under different handling and storage conditions for metabonomics studies. J. Chromatogr. A 1189, 314–322. https://doi.org/10.1016/j.chroma.2007.10.066.

Gika, H.G., Theodoridis, G.A., Plumb, R.S., Wilson, I.D., 2014. Current practice of liquid chromatography-mass spectrometry in metabolomics and metabonomics. J. Pharmaceut. Biomed. Anal. 87, 12–25. https://doi.org/10.1016/j.jpba.2013.06.032.

Giskeødegård, G.F., Davies, S.K., Revell, V.L., Keun, H., Skene, D.J., 2015. Diurnal rhythms in the human urine metabolome during sleep and total sleep deprivation. Sci. Rep. 5, 14843. https://doi.org/10.1038/srep14843.

Gordon, W.C., Bazan, N.G., 2013. Mediator lipidomics in ophthalmology: targets for modulation in inflammation, neuroprotection and nerve regeneration. Curr. Eye Res. 38, 995–1005. https://doi.org/10.3109/02713683.2013.827211.

Hebels, D.G.A.J., Georgiadis, P., Keun, H.C., Athersuch, T.J., Vineis, P., Vermeulen, R., Portengen, L., Bergdahl, I.A., Hallmans, G., Palli, D., Bendinelli, B., Krogh, V., Tumino, R., Sacerdote, C., Panico, S., Kleinjans, J.C., de Kok, T.M., Smith, M.T., Kyrtopoulos, S.A., EnviroGenomarkers Project Consortium, 2013. Performance in omics analyses of blood samples in long-term storage: opportunities for the exploitation of existing biobanks in environmental health research. Environ. Health Perspect. 121, 480–487. https://doi.org/10.1289/ehp.1205657.

Holekamp, N.M., 2010. The vitreous gel: more than meets the eye. Am. J. Ophthalmol. 149, 32–36. e1. https://doi.org/10.1016/j.ajo.2009.07.036.

Holmes, E., Wijeyesekera, A., Taylor-Robinson, S.D., Nicholson, J.K., 2015. The promise of metabolic phenotyping in gastroenterology and hepatology. Nat. Rev. Gastroenterol. Hepatol. 12, 458–471. https://doi.org/10.1038/nrgastro.2015.114.

Holmes, E., Nicholson, J.K., Darzi, A.W., Lindon, J.C., 2016. Metabolic phenotyping in personalized and public healthcare. Metabolic Phenotyping in Personalized and Public Healthcare. https://doi.org/10.1016/C2013-0-13640-9.

Hopiavuori, B.R., Agbaga, M.-P., Brush, R.S., Sullivan, M.T., Sonntag, W.E., Anderson, R.E., 2017. Regional changes in CNS and retinal glycerophospholipid profiles with age: a molecular blueprint. J. Lipid Res. 58, 668–680. https://doi.org/10.1194/jlr.M070714.

Hu, T., Zhang, J.-L., 2018. Mass-spectrometry-based lipidomics. J. Separ. Sci. 41, 351–372. https://doi.org/10.1002/jssc.201700709.

Hurst, J.L., West, R.S., 2010. Taming anxiety in laboratory mice. Nat. Methods 7, 825–826. https://doi.org/10.1038/nmeth.1500.

Inouye, M., Kettunen, J., Soininen, P., Silander, K., Ripatti, S., Kumpula, L.S., Hämäläinen, E., Jousilahti, P., Kangas, A.J., Männistö, S., Savolainen, M.J., Jula, A., Leiviskä, J., Palotie, A., Salomaa, V., Perola, M., Ala-Korpela, M., Peltonen, L., 2010. Metabonomic, transcriptomic, and genomic variation of a population cohort. Mol. Syst. Biol. 6, 441. https://doi.org/10.1038/msb.2010.93.

Jafari, M., Ansari-Pour, N., Azimzadeh, S., Mirzaie, M., 2017. A logic-based dynamic modeling approach to explicate the evolution of the central dogma of molecular biology. PloS One 12, e0189922. https://doi.org/10.1371/journal.pone.0189922.

Jarmusch, A.K., Pirro, V., Baird, Z., Hattab, E.M., Cohen-Gadol, A.A., Cooks, R.G., 2016. Lipid and metabolite profiles of human brain tumors by desorption electrospray ionization-MS. Proc. Natl. Acad. Sci. Unit. States Am. 113, 1486–1491. https://doi.org/10.1073/pnas.1523306113.

Jobard, E., Trédan, O., Postoly, D., André, F., Martin, A.-L., Elena-Herrmann, B., Boyault, S., 2016. A systematic evaluation of blood serum and plasma pre-analytics for metabolomics cohort studies. Int. J. Mol. Sci. 17, 2035. https://doi.org/10.3390/ijms17122035.

Jové, M., Portero-Otín, M., Naudí, A., Ferrer, I., Pamplona, R., 2014. Metabolomics of human brain aging and age-related neurodegenerative diseases. J. Neuropathol. Exp. Neurol. 73, 640–657. https://doi.org/10.1097/NEN.0000000000000091.

Kaarniranta, K., Salminen, A., Haapasalo, A., Soininen, H., Hiltunen, M., 2011. Age-related macular degeneration (AMD): Alzheimer's disease in the eye? J. Alzheimers. Dis. 24, 615–631. https://doi.org/10.3233/JAD-2011-101908.

Kamburov, A., Cavill, R., Ebbels, T.M.D., Herwig, R., Keun, H.C., 2011. Integrated pathway-level analysis of transcriptomics and metabolomics data with IMPaLA. Bioinformatics 27, 2917–2918. https://doi.org/10.1093/bioinformatics/btr499.

Kapoore, R.V., Vaidyanathan, S., 2016. Towards quantitative mass spectrometry-based metabolomics in microbial and mammalian systems. Philos. Trans. A. Math. Phys. Eng. Sci. 374. https://doi.org/10.1098/rsta.2015.0363.

Kell, D.B., Oliver, S.G., 2016. The metabolome 18 years on: a concept comes of age. Metabolomics 12, 148. https://doi.org/10.1007/s11306-016-1108-4.

Kelly, A.D., Breitkopf, S.B., Yuan, M., Goldsmith, J., Spentzos, D., Asara, J.M., 2011. Metabolomic Profiling from Formalin-Fixed, Paraffin-Embedded Tumor Tissue Using Targeted LC/MS/MS: application in Sarcoma. PloS One 6, e25357. https://doi.org/10.1371/journal.pone.0025357.

Kersten, E., Paun, C.C., Schellevis, R.L., Hoyng, C.B., Delcourt, C., Lengyel, I., Peto, T., Ueffing, M., Klaver, C.C.W., Dammeier, S., den Hollander, A.I., de Jong, E.K., 2018. Systemic and ocular fluid compounds as potential biomarkers in age-related macular degeneration. Surv. Ophthalmol. 63, 9–39. https://doi.org/10.1016/j.survophthal.2017.05.003.

Khamis, M.M., Adamko, D.J., El-Aneed, A., 2017. Mass spectrometric based approaches in urine metabolomics and biomarker discovery. Mass Spectrom. Rev. 36, 115–134. https://doi.org/10.1002/mas.21455.

Kohler, I., Verhoeven, A., Derks, R.J., Giera, M., 2016. Analytical pitfalls and challenges in clinical metabolomics. Bioanalysis 8, 1509–1532. https://doi.org/10.4155/bio-2016-0090.

Kosicek, M., Hecimovic, S., 2013. Phospholipids and Alzheimer's disease: alterations, mechanisms and potential biomarkers. Int. J. Mol. Sci. 14, 1310–1322. https://doi.org/10.3390/ijms14011310.

Kraft, M.L., Klitzing, H.A., 2014. Imaging lipids with secondary ion mass spectrometry. Biochim. Biophys. Acta Mol. Cell Biol. Lipids 1841, 1108–1119. https://doi.org/10.1016/j.bbalip.2014.03.003.

Kumar, N., Hoque, M.A., Shahjaman, M., Islam, S.M.S., Mollah, M.N.H., 2017. Metabolomic biomarker identification in presence of outliers and missing values. BioMed Res. Int. 2017, 1–11. https://doi.org/10.1155/2017/2437608.

Kurihara, T., Westenskow, P.D., Gantner, M.L., Usui, Y., Schultz, A., Bravo, S., Aguilar, E., Wittgrove, C., Friedlander, M.S., Paris, L.P., Chew, E., Siuzdak, G., Friedlander, M., 2016. Hypoxia-induced metabolic stress in retinal pigment epithelial cells is sufficient to induce photoreceptor degeneration. Elife 5. https://doi.org/10.7554/eLife.14319.

Laatikainen, L., Tolppanen, E.M., Harju, H., 1985. Epidemiology of rhegmatogenous retinal detachment in a Finnish population. Acta Ophthalmol. 63, 59–64.

Laíns, I., Duarte, D., Barros, A.S., Martins, A.S., Gil, J., Miller, J.B., Marques, M., Mesquita, T., Kim, I.K., Da Luz Cachulo, M., Vavvas, D., Carreira, I.M., Murta, J.N., Silva, R., Miller, J.W., Husain, D., Gil, A.M., 2017a. Human plasma metabolomics in age-related macular degeneration (AMD) using nuclear magnetic resonance spectroscopy. PloS One 12. https://doi.org/10.1371/journal.pone.0177749.

Laíns, I., Kelly, R.S., Miller, J.B., Silva, R., Vavvas, D.G., Kim, I.K., Murta, J.N., Lasky-Su, J., Miller, J.W., Husain, D., 2017b. Human plasma metabolomics study across all stages of age-related macular degeneration identifies potential lipid biomarkers. Ophthalmology. https://doi.org/10.1016/j.ophtha.2017.08.008.

Lam, S.M., Tong, L., Duan, X., Petznick, A., Wenk, M.R., Shui, G., 2014. Extensive characterization of human tear fluid collected using different techniques unravels the presence of novel lipid amphiphiles. J. Lipid Res. 55, 289–298. https://doi.org/10.1194/jlr.M044826.

Lau, J.C.M., Linsenmeier, R.A., 2012. Oxygen consumption and distribution in the Long-Evans rat retina. Exp. Eye Res. 102, 50–58. https://doi.org/10.1016/j.exer.2012.07.004.

Li, X., Luo, X., Lu, X., Duan, J., Xu, G., 2011. Metabolomics study of diabetic retinopathy using gas chromatography–mass spectrometry: a comparison of stages and subtypes diagnosed by Western and Chinese medicine. Mol. Biosyst. 7, 2228. https://doi.org/10.1039/c0mb00341g.

Li, M., Li, H., Jiang, P., Liu, X., Xu, D., Wang, F., 2014. Investigating the pathological processes of rhegmatogenous retinal detachment and proliferative vitreoretinopathy with metabolomics analysis. Mol. Biosyst. 10, 1055. https://doi.org/10.1039/c3mb70386j.

Li, M., Zhang, X., Liao, N., Ye, B., Peng, Y., Ji, Y., Wen, F., 2016. Analysis of the serum lipid profile in polypoidal choroidal vasculopathy. Sci. Rep. 6, 38342. https://doi.org/10.1038/srep38342.

Lind, M.V., Savolainen, O.I., Ross, A.B., 2016. The use of mass spectrometry for analysing metabolite biomarkers in epidemiology: methodological and statistical considerations for application to large numbers of biological samples. Eur. J. Epidemiol. 31, 717–733. https://doi.org/10.1007/s10654-016-0166-2.

Linton, J.D., Holzhausen, L.C., Babai, N., Song, H., Miyagishima, K.J., Stearns, G.W., Lindsay, K., Wei, J., Chertov, A.O., Peters, T.A., Caffe, R., Pluk, H., Seeliger, M.W., Tanimoto, N., Fong, K., Bolton, L., Kuok, D.L.T., Sweet, I.R., Bartoletti, T.M., Radu, R.A., Travis, G.H., Zagotta, W.N., Townes-Anderson, E., Parker, E., Van der Zee, C.E.E.M., Sampath, A.P., Sokolov, M., Thoreson, W.B., Hurley, J.B., 2010. Flow of energy in the outer retina in darkness and in light. Proc. Natl. Acad. Sci. U. S. A 107, 8599–8604. https://doi.org/10.1073/pnas.1002471107.

Locci, E., Scano, P., Rosa, M.F., Nioi, M., Noto, A., Atzori, L., Demontis, R., De-Giorgio, F., d'Aloja, E., 2014. A Metabolomic Approach to Animal Vitreous Humor Topographical Composition: a Pilot Study. PloS One 9, e97773. https://doi.org/10.1371/journal.pone.0097773.

Lockyer, N.P., 2014. Secondary ion mass spectrometry imaging of biological cells and tissues. In: Methods in Molecular Biology. Clifton, N.J., pp. 707–732. https://doi.org/10.1007/978-1-62703-776-1_32.

Lorés-Motta, L., de Jong, E.K., den Hollander, A.I., 2018. Exploring the use of molecular biomarkers for precision medicine in age-related macular degeneration. Mol. Diagn. Ther. 22, 315–343. https://doi.org/10.1007/s40291-018-0332-1.

Luan, Y., Ou, J., Kunze, V.P., Qiao, F., Wang, Y., Wei, L., Li, W., Xie, Z., 2018. Integrated transcriptomic and metabolomic analysis reveals adaptive changes of hibernating retinas. J. Cell. Physiol. 233, 1434–1445. https://doi.org/10.1002/jcp.26030.

Luo, D.-G., Xue, T., Yau, K.-W., 2008. How Vision Begins: an Odyssey. 105. pp. 9855–9862. doi.org. https://doi.org/10.1073/pnas.0708405105.

Ly, A., Schöne, C., Becker, M., Rattke, J., Meding, S., Aichler, M., Suckau, D., Walch, A., Hauck, S.M., Ueffing, M., 2015. High-resolution MALDI mass spectrometric imaging of lipids in the mammalian retina. Histochem. Cell Biol. 143, 453–462. https://doi.org/10.1007/s00418-014-1303-1.

Mains, J., Tan, L.E., Zhang, T., Young, L., Shi, R., Wilson, C., 2012. Species variation in small molecule components of animal vitreous. Invest. Ophthalmol. Vis. Sci. 53, 4778–4786. https://doi.org/10.1167/iovs.12-9998.

Mal, M., 2016. Noninvasive metabolic profiling for painless diagnosis of human diseases and disorders. Futur. Sci. OA 2 fsoa-2015-0014. https://doi.org/10.4155/fsoa-2015-0014.

Manolio, T.A., Collins, F.S., Cox, N.J., Goldstein, D.B., Hindorff, L.A., Hunter, D.J., McCarthy, M.I., Ramos, E.M., Cardon, L.R., Chakravarti, A., Cho, J.H., Guttmacher, A.E., Kong, A., Kruglyak, L., Mardis, E., Rotimi, C.N., Slatkin, M., Valle, D., Whittemore, A.S., Boehnke, M., Clark, A.G., Eichler, E.E., Gibson, G., Haines, J.L., Mackay, T.F.C., McCarroll, S.A., Visscher, P.M., 2009. Finding the missing heritability of complex diseases. Nature 461, 747–753. https://doi.org/10.1038/nature08494.

Mapstone, M., Cheema, A.K., Fiandaca, M.S., Zhong, X., Mhyre, T.R., MacArthur, L.H., Hall, W.J., Fisher, S.G., Peterson, D.R., Haley, J.M., Nazar, M.D., Rich, S.A., Berlau, D.J., Peltz, C.B., Tan, M.T., Kawas, C.H., Federoff, H.J., 2014. Plasma phospholipids identify antecedent memory impairment in older adults. Nat. Med. 20, 415–418. https://doi.org/10.1038/nm.3466.

Mapstone, M., Lin, F., Nalls, M.A., Cheema, A.K., Singleton, A.B., Fiandaca, M.S., Federoff, H.J., 2017. What success can teach us about failure: the plasma metabolome of older adults with superior memory and lessons for Alzheimer's disease. Neurobiol. Aging 51, 148–155. https://doi.org/10.1016/j.neurobiolaging.2016.11.007.

Marco-Ramell, A., Palau-Rodriguez, M., Alay, A., Tulipani, S., Urpi-Sarda, M., Sanchez-Pla, A., Andres-Lacueva, C., 2018. Evaluation and comparison of bioinformatic tools for the enrichment analysis of metabolomics data. BMC Bioinf. 19, 1. https://doi.org/10.1186/s12859-017-2006-0.

Markley, J.L., Brüschweiler, R., Edison, A.S., Eghbalnia, H.R., Powers, R., Raftery, D., Wishart, D.S., 2017. The future of NMR-based metabolomics. Curr. Opin. Biotechnol. 43, 34–40. https://doi.org/10.1016/j.copbio.2016.08.001.

Marshall, D.D., Powers, R., 2017. Beyond the paradigm: combining mass spectrometry and nuclear magnetic resonance for metabolomics. Prog. Nucl. Magn. Reson. Spectrosc. 100, 1–16. https://doi.org/10.1016/j.pnmrs.2017.01.001.

Midelfart, A., 2009. Metabonomics–a new approach in ophthalmology. Acta Ophthalmol. 87, 697–703.

Mitry, D., Charteris, D.G., Yorston, D., Siddiqui, M.A.R., Campbell, H., Murphy, A.-L., Fleck, B.W., Wright, A.F., Singh, J., Scottish, R.D., Study Group, 2010. The epidemiology and socioeconomic associations of retinal detachment in scotland: a two-year prospective population-based study. Investig. Opthalmology Vis. Sci. 51, 4963. https://doi.org/10.1167/iovs.10-5400.

Monteiro, M.S., Carvalho, M., Bastos, M.L., Guedes de Pinho, P., 2013. Metabolomics analysis for biomarker discovery: advances and challenges. Curr. Med. Chem. 20, 257–271.

Murphy, R.C., Hankin, J.A., Barkley, R.M., Zemski Berry, K.A., 2011. MALDI imaging of lipids after matrix sublimation/deposition. Biochim. Biophys. Acta 1811, 970–975. https://doi.org/10.1016/j.bbalip.2011.04.012.

Narayanan, S.P., Rojas, M., Suwanpradid, J., Toque, H.A., Caldwell, R.W., Caldwell, R.B., 2013. Arginase in retinopathy. Prog. Retin. Eye Res. 36, 260–280. https://doi.org/10.1016/j.preteyeres.2013.06.002.

Naru, J., Aggarwal, R., Mohanty, A.K., Singh, U., Bansal, D., Kakkar, N., Agnihotri, N., 2017. Identification of differentially expressed proteins in retinoblastoma tumors using mass spectrometry-based comparative proteomic approach. J. Proteomics 159, 77–91. https://doi.org/10.1016/j.jprot.2017.02.006.

Nelson, C.J., Otis, J.P., Martin, S.L., Carey, H.V., 2009. Analysis of the hibernation cycle using LC-MS-based metabolomics in ground squirrel liver. Physiol. Genom. 37, 43–51. https://doi.org/10.1152/physiolgenomics.90323.2008.

Nicholson, J., Holmes, E., Kinross, J.M., Darzi, A.W., Takats, Z., Lindon, J.C., 2012a. Metabolic phenotyping in clinical and surgical environments. Nature 491, 384–392.

Nicholson, J.K., Everett, J.R., Lindon, J.C., 2012b. Longitudinal pharmacometabonomics for predicting patient responses to therapy: drug metabolism, toxicity and efficacy. Expet Opin. Drug Metabol. Toxicol. 8, 135–139.

Nirmalan, N.J., Harnden, P., Selby, P.J., Banks, R.E., 2008. Mining the archival formalin-fixed paraffin-embedded tissue proteome: opportunities and challenges. Mol. Biosyst. 4, 712–720. https://doi.org/10.1039/b800098k.

Nishtala, K., Pahuja, N., Shetty, R., Nuijts, R.M.M.A., Ghosh, A., 2016. Tear biomarkers for keratoconus. Eye Vis 3, 19. https://doi.org/10.1186/s40662-016-0051-9.

Nyamundanda, G., Gormley, I.C., Fan, Y., Gallagher, W.M., Brennan, L., 2013. MetSizeR: selecting the optimal sample size for metabolomic studies using an analysis based approach. BMC Bioinf. 14, 338. https://doi.org/10.1186/1471-2105-14-338.

Obuchowski, N.A., Lieber, M.L., Wians, F.H., 2004. ROC curves in clinical chemistry: uses, misuses, and possible solutions. Clin. Chem. 50, 1118–1125. https://doi.org/10.1373/clinchem.2004.031823.

Ohno-Matsui, K., 2011. Parallel findings in age-related macular degeneration and Alzheimer's disease. Prog. Retin. Eye Res. 30, 217–238. https://doi.org/10.1016/j.preteyeres.2011.02.004.

Osborn, M.P., Park, Y., Parks, M.B., Burgess, L.G., Uppal, K., Lee, K., Jones, D.P., Brantley, M.A., 2013. Metabolome-wide association study of neovascular age-related macular degeneration. PloS One 8, e72737.

Osborne, N.N., Casson, R.J., Wood, J.P., Chidlow, G., Graham, M., Melena, J., 2004. Retinal ischemia: mechanisms of damage and potential therapeutic strategies. Prog. Retin. Eye Res. 23, 91–147. https://doi.org/10.1016/J.PRETEYERES.2003.12.001.

Paris, L.P., Johnson, C.H., Aguilar, E., Usui, Y., Cho, K., Hoang, L.T., Feitelberg, D., Benton, H.P., Westenskow, P.D., Kurihara, T., Trombley, J., Tsubota, K., Ueda, S., Wakabayashi, Y., Patti, G.J., Ivanisevic, J., Siuzdak, G., Friedlander, M., 2016. Global metabolomics reveals metabolic dysregulation in ischemic retinopathy. Metabolomics 12, 15. https://doi.org/10.1007/s11306-015-0877-5.

Pastor, J.C., Rojas, J., Pastor-Idoate, S., Di Lauro, S., Gonzalez-Buendia, L., Delgado-Tirado, S., 2016. Proliferative vitreoretinopathy: a new concept of disease pathogenesis and practical consequences. Prog. Retin. Eye Res. 51, 125–155. https://doi.org/10.1016/j.preteyeres.2015.07.005.

Patel, C.J., Kerr, J., Thomas, D.C., Mukherjee, B., Ritz, B., Chatterjee, N., Jankowska, M., Madan, J., Karagas, M.R., McAllister, K.A., Mechanic, L.E., Fallin, M.D., Ladd-Acosta, C., Blair, I.A., Teitelbaum, S.L., Amos, C.I., 2017. Opportunities and challenges for environmental exposure assessment in population-based studies. Cancer Epidemiol. Biomark. Prev. 26, 1370–1380. https://doi.org/10.1158/1055-9965.EPI-17-0459.

Patti, G.J., Yanes, O., Siuzdak, G., 2012. Innovation: metabolomics: the apogee of the omics trilogy. Nat. Rev. Mol. Cell Biol. 13, 263–269. https://doi.org/10.1038/nrm3314.

Petras, D., Jarmusch, A.K., Dorrestein, P.C., 2017. From single cells to our planet-recent advances in using mass spectrometry for spatially resolved metabolomics. Curr. Opin. Chem. Biol. 36, 24–31. https://doi.org/10.1016/j.cbpa.2016.12.018.

Pieragostino, D., D'Alessandro, M., di Ioia, M., Di Ilio, C., Sacchetta, P., Del Boccio, P., 2015. Unraveling the molecular repertoire of tears as a source of biomarkers: beyond ocular diseases. Proteonomics Clin. Appl. 9, 169–186. https://doi.org/10.1002/prca.201400084.

Pietrowska, K., Dmuchowska, D.A., Samczuk, P., Kowalczyk, T., Krasnicki, P., Wojnar, M., Skowronska, A., Mariak, Z., Kretowski, A., Ciborowski, M., 2017. LC-MS-Based metabolic fingerprinting of aqueous humor. J. Anal. Methods Chem. 2017, 1–13. https://doi.org/10.1155/2017/6745932.

Pietrowska, K., Dmuchowska, D.A., Krasnicki, P., Bujalska, A., Samczuk, P., Parfieniuk, E., Kowalczyk, T., Wojnar, M., Mariak, Z., Kretowski, A., Ciborowski, M., 2018. An exploratory LC-MS-based metabolomics study reveals differences in aqueous humor composition between diabetic and non-diabetic patients with cataract. Electrophoresis 39, 1233–1240. https://doi.org/10.1002/elps.201700411.

Pilgrim, M.G., Lengyel, I., Lanzirotti, A., Newville, M., Fearn, S., Emri, E., Knowles, J.C., Messinger, J.D., Read, R.W., Guidry, C., Curcio, C.A., 2017. Subretinal pigment epithelial deposition of drusen components including hydroxyapatite in a primary cell culture model. Investig. Opthalmology Vis. Sci. 58, 708. https://doi.org/10.1167/iovs.16-21060.

Proitsi, P., Kim, M., Whiley, L., Simmons, A., Sattlecker, M., Velayudhan, L., Lupton, M.K., Soininen, H., Kloszewska, I., Mecocci, P., Tsolaki, M., Vellas, B., Lovestone, S., Powell, J.F., Dobson, R.J.B., Legido-Quigley, C., 2017. Association of blood lipids with Alzheimer's disease: a comprehensive lipidomics analysis. Alzheimer's Dementia 13, 140–151. https://doi.org/10.1016/j.jalz.2016.08.003.

Psychogios, N., Hau, D.D., Peng, J., Guo, A.C., Mandal, R., Bouatra, S., Sinelnikov, I., Krishnamurthy, R., Eisner, R., Gautam, B., Young, N., Xia, J., Knox, C., Dong, E., Huang, P., Hollander, Z., Pedersen, T.L., Smith, S.R., Bamforth, F., Greiner, R., McManus, B., Newman, J.W., Goodfriend, T., Wishart, D.S., 2011. The Human Serum Metabolome. PloS One 6, e16957. https://doi.org/10.1371/journal.pone.0016957.

Putri, S.P., Yamamoto, S., Tsugawa, H., Fukusaki, E., 2013. Current metabolomics: technological advances. J. Biosci. Bioeng. 116, 9–16. https://doi.org/10.1016/j.jbiosc.2013.01.004.

Rantamäki, A.H., Seppänen-Laakso, T., Oresic, M., Jauhiainen, M., Holopainen, J.M., 2011. Human tear fluid lipidome: from composition to function. PloS One 6, e19553. https://doi.org/10.1371/journal.pone.0019553.

Romero, P., Wagg, J., Green, M.L., Kaiser, D., Krummenacker, M., Karp, P.D., 2005. Computational prediction of human metabolic pathways from the complete human genome. Genome Biol. 6, R2. https://doi.org/10.1186/gb-2004-6-1-r2.

Rosato, A., Tenori, L., Cascante, M., De Atauri Carulla, P.R., Martins dos Santos, V.A.P., Saccenti, E., 2018. From correlation to causation: analysis of metabolomics data using systems biology approaches. Metabolomics 14, 37. https://doi.org/10.1007/s11306-018-1335-y.

Rowan, S., Jiang, S., Korem, T., Szymanski, J., Chang, M.-L., Szelog, J., Cassalman, C., Dasuri, K., McGuire, C., Nagai, R., Du, X.-L., Brownlee, M., Rabbani, N., Thornalley, P.J., Baleja, J.D., Deik, A.A., Pierce, K.A., Scott, J.M., Clish, C.B., Smith, D.E., Weinberger, A., Avnit-Sagi, T., Lotan-Pompan, M., Segal, E., Taylor, A., 2017. Involvement of a gut–retina axis in protection against dietary glycemia-induced age-related macular degeneration. Proc. Natl. Acad. Sci. Unit. States Am. 114, E4472–E4481. https://doi.org/10.1073/pnas.1702302114.

Rowe, J.A., Erie, J.C., Baratz, K.H., Hodge, D.O., Gray, D.T., Butterfield, L., Robertson, D.M., 1999. Retinal detachment in olmsted county, Minnesota, 1976 through 1995. Ophthalmology 106, 154–159. https://doi.org/10.1016/S0161-6420(99)90018-0.

Rucker, J.C., Biousse, V., Mao, H., Sandbach, J., Constantinidis, I., Newman, N.J., 2003. Detection of lactate in the human vitreous body using proton magnetic resonance spectroscopy. Arch. Ophthalmol. 121, 909–911. https://doi.org/10.1001/archopht.121.6.909.

Rusilowicz, M., Dickinson, M., Charlton, A., O'Keefe, S., Wilson, J., 2016. A batch correction method for liquid chromatography–mass spectrometry data that does not depend on quality control samples. Metabolomics 12, 56. https://doi.org/10.1007/s11306-016-0972-2.

Scalbert, A., Brennan, L., Fiehn, O., Hankemeier, T., Kristal, B.S., van Ommen, B., Pujos-Guillot, E., Verheij, E., Wishart, D., Wopereis, S., 2009. Mass-spectrometry-based

metabolomics: limitations and recommendations for future progress with particular focus on nutrition research. Metabolomics 5, 435–458. https://doi.org/10.1007/s11306-009-0168-0.

Scalbert, A., Brennan, L., Manach, C., Andres-Lacueva, C., Dragsted, L.O., Draper, J., Rappaport, S.M., van der Hooft, J.J.J., Wishart, D.S., 2014. The food metabolome: a window over dietary exposure. Am. J. Clin. Nutr. 99, 1286–1308. https://doi.org/10.3945/ajcn.113.076133.

Schmidt, S.Y., Berson, E.L., 1980. Postmortem metabolic capacity of photoreceptor cells in human and rat retinas. Invest. Ophthalmol. Vis. Sci. 19, 1274–1280.

Shui, Y.-B., Holekamp, N.M., Kramer, B.C., Crowley, J.R., Wilkins, M.A., Chu, F., Malone, P.E., Mangers, S.J., Hou, J.H., Siegfried, C.J., Beebe, D.C., 2009. The gel state of the vitreous and ascorbate-dependent oxygen consumption: relationship to the etiology of nuclear cataracts. Arch. Ophthalmol. 127, 475–482. https://doi.org/10.1001/archophthalmol.2008.621.

Singh, R.P., Habbu, K.A., Bedi, R., Silva, F.Q., Ehlers, J.P., Schachat, A.P., Sears, J.E., Srivastava, S.K., Kaiser, P.K., Yuan, A., 2017. A retrospective study of the influence of the vitreomacular interface on macular oedema secondary to retinal vein occlusion. Br. J. Ophthalmol. 101, 1340–1345. https://doi.org/10.1136/bjophthalmol-2016-309747.

Sivak, J.M., 2013. The aging eye: common degenerative mechanisms between the Alzheimer's brain and retinal disease. Investig. Opthalmology Vis. Sci. 54, 871. https://doi.org/10.1167/iovs.12-10827.

Slupsky, C.M., Rankin, K.N., Wagner, J., Fu, H., Chang, D., Weljie, A.M., Saude, E.J., Lix, B., Adamko, D.J., Shah, S., Greiner, R., Sykes, B.D., Marrie, T.J., 2007. Investigations of the effects of gender, diurnal variation, and age in human urinary metabolomic profiles. Anal. Chem. 79, 6995–7004. https://doi.org/10.1021/ac0708588.

Sobrin, L., Seddon, J.M., 2013. Nature and nurture- genes and environment- predict onset and progression of macular degeneration. Prog. Retin. Eye Res. https://doi.org/10.1016/j.preteyeres.2013.12.004.

Solberg, R., Escobar, J., Arduini, A., Torres-Cuevas, I., Lahoz, A., Sastre, J., Saugstad, O.D., Vento, M., Kuligowski, J., Quintás, G., 2013. Metabolomic Analysis of the Effect of Postnatal Hypoxia on the Retina in a Newly Born Piglet Model. PloS One 8, e66540. https://doi.org/10.1371/journal.pone.0066540.

Stem, M.S., Gardner, T.W., 2013. Neurodegeneration in the pathogenesis of diabetic retinopathy: molecular mechanisms and therapeutic implications. Curr. Med. Chem. 20, 3241–3250.

Suhre, K., Gieger, C., 2012. Genetic variation in metabolic phenotypes: study designs and applications. Nat. Rev. Genet. 13, 759–769.

Suhre, K., Meisinger, C., Döring, A., Altmaier, E., Belcredi, P., Gieger, C., Chang, D., Milburn, M.V., Gall, W.E., Weinberger, K.M., Mewes, H.-W., Hrabé de Angelis, M., Wichmann, H.-E., Kronenberg, F., Adamski, J., Illig, T., 2010. Metabolic footprint of diabetes: a multiplatform metabolomics study in an epidemiological setting. PloS One 5, e13953. https://doi.org/10.1371/journal.pone.0013953.

Suhre, K., Shin, S.-Y., Petersen, A.-K., Mohney, R.P., Meredith, D., Wägele, B., Altmaier, E., CARDIoGRAM, Deloukas, P., Erdmann, J., Grundberg, E., Hammond, C.J., de Angelis, M.H., Kastenmüller, G., Köttgen, A., Kronenberg, F., Mangino, M., Meisinger, C., Meitinger, T., Mewes, H.-W., Milburn, M.V., Prehn, C., Raffler, J., Ried, J.S., Römisch-Margl, W., Samani, N.J., Small, K.S., Wichmann, H.-E., Zhai, G., Illig, T., Spector, T.D., Adamski, J., Soranzo, N., Gieger, C., 2011. Human metabolic individuality in biomedical and pharmaceutical research. Nature 477, 54–60. https://doi.org/10.1038/nature10354.

Sun, Y.V., Hu, Y.-J., 2016. Integrative analysis of multi-omics data for discovery and functional studies of complex human diseases. In: Advances in Genetics, pp. 147–190. https://doi.org/10.1016/bs.adgen.2015.11.004.

Sun, N., Ly, A., Meding, S., Witting, M., Hauck, S.M., Ueffing, M., Schmitt-Kopplin, P., Aichler, M., Walch, A., 2014. High-resolution metabolite imaging of light and dark treated retina using MALDI-FTICR mass spectrometry. Proteomics 14, 913–923. https://doi.org/10.1002/pmic.201300407.

Szymańska, E., Saccenti, E., Smilde, A.K., Westerhuis, J.A., 2012. Double-check: validation of diagnostic statistics for PLS-DA models in metabolomics studies. Metabolomics 8, 3–16. https://doi.org/10.1007/s11306-011-0330-3.

Takeda, I., Stretch, C., Barnaby, P., Bhatnager, K., Rankin, K., Fu, H., Weljie, A., Jha, N., Slupsky, C., 2009. Understanding the human salivary metabolome. NMR Biomed. 22, 577–584. https://doi.org/10.1002/nbm.1369.

Tan, S.Z., Begley, P., Mullard, G., Hollywood, K.A., Bishop, P.N., 2016a. Introduction to metabolomics and its applications in ophthalmology. Eye 30, 773–783. https://doi.org/10.1038/eye.2016.37.

Tan, S.Z., Mullard, G., Hollywood, K.A., Dunn, W.B., Bishop, P.N., 2016b. Characterisation of the metabolome of ocular tissues and post-mortem changes in the rat retina. Exp. Eye Res. 149, 8–15. https://doi.org/10.1016/j.exer.2016.05.019.

Thompson, R.B., Reffatto, V., Bundy, J.G., Kortvely, E., Flinn, J.M., Lanzirotti, A., Jones, E.A., McPhail, D.S., Fearn, S., Boldt, K., Ueffing, M., Ratu, S.G.S., Pauleikhoff, L., Bird, A.C., Lengyel, I., 2015. Identification of hydroxyapatite spherules provides new insight into subretinal pigment epithelial deposit formation in the aging eye. Proc. Natl. Acad. Sci. Unit. States Am. 112, 1565–1570. https://doi.org/10.1073/pnas.1413347112.

Ting, D.S.W., Tan, K.-A., Phua, V., Tan, G.S.W., Wong, C.W., Wong, T.Y., 2016. Biomarkers of diabetic retinopathy. Curr. Diabetes Rep. 16, 125. https://doi.org/10.1007/s11892-016-0812-9.

Tognarelli, J.M., Dawood, M., Shariff, M.I.F., Grover, V.P.B., Crossey, M.M.E., Cox, I.J., Taylor-Robinson, S.D., McPhail, M.J.W., 2015. Magnetic resonance spectroscopy: principles and techniques: lessons for clinicians. J. Clin. Exp. Hepatol. 5, 320–328. https://doi.org/10.1016/j.jceh.2015.10.006.

Tolstikov, V., 2016. Metabolomics: bridging the gap between pharmaceutical development and population health. Metabolites 6, 20. https://doi.org/10.3390/metabo6030020.

Törnquist, R., Stenkula, S., Törnquist, P., 1987. Retinal detachment. A study of a population-based patient material in Sweden 1971-1981. I. Epidemiology. Acta Ophthalmol. 65, 213–222.

Triba, M.N., Le Moyec, L., Amathieu, R., Goossens, C., Bouchemal, N., Nahon, P., Rutledge, D.N., Savarin, P., 2015. PLS/OPLS models in metabolomics: the impact of permutation of dataset rows on the K-fold cross-validation quality parameters. Mol. Biosyst. 11, 13–19. https://doi.org/10.1039/c4mb00414k.

Trivedi, D.K., Hollywood, K.A., Goodacre, R., 2017. Metabolomics for the masses: the future of metabolomics in a personalized world. New Horizons Transl. Med. https://doi.org/10.1016/j.nhtm.2017.06.001.

Tseng, W., Cortez, R.T., Ramirez, G., Stinnett, S., Jaffe, G.J., 2004. Prevalence and risk factors for proliferative vitreoretinopathy in eyes with rhegmatogenous retinal detachment but no previous vitreoretinal surgery. Am. J. Ophthalmol. 137, 1105–1115. https://doi.org/10.1016/j.ajo.2004.02.008.

van Iterson, M., 't Hoen, P.A.C., Pedotti, P., Hooiveld, G.J.E.J., den Dunnen, J.T., van Ommen, G.J.B., Boer, J.M., Menezes, R.X., 2009. Relative power and sample size analysis on gene expression profiling data. BMC Genomics 10, 439. https://doi.org/10.1186/1471-2164-10-439.

Viant, M.R., Kurland, I.J., Jones, M.R., Dunn, W.B., 2017. How close are we to complete annotation of metabolomes? Curr. Opin. Chem. Biol. 36, 64–69. https://doi.org/10.1016/J.CBPA.2017.01.001.

Wang, H.-Y., Chu, X., Zhao, Z.-X., He, X.-S., Guo, Y.-L., 2011a. Analysis of low molecular weight compounds by MALDI-FTICR-MS. J. Chromatogr. B 879, 1166–1179. https://doi.org/10.1016/j.jchromb.2011.03.037.

Wang, X., Sun, H., Zhang, A., Wang, P., Han, Y., 2011b. Ultra-performance liquid chromatography coupled to mass spectrometry as a sensitive and powerful technology for metabolomic studies. J. Separ. Sci. 34, 3451–3459. https://doi.org/10.1002/jssc.201100333.

Wang, C., Qin, N., Zhu, M., Chen, M., Xie, K., Cheng, Y., Dai, J., Liu, J., Xia, Y., Ma, H., Jin, G., Amos, C.I., Hu, Z., Lin, D., Shen, H., 2017. Metabolome-wide association study identified the association between a circulating polyunsaturated fatty acids variant rs174548 and lung cancer. Carcinogenesis 38, 1147–1154. https://doi.org/10.1093/carcin/bgx084.

Wehrens, R., Hageman, J.A., van Eeuwijk, F., Kooke, R., Flood, P.J., Wijnker, E., Keurentjes, J.J.B., Lommen, A., van Eekelen, H.D.L.M., Hall, R.D., Mumm, R., de Vos, R.C.H., 2016. Improved batch correction in untargeted MS-based metabolomics. Metabolomics 12, 88. https://doi.org/10.1007/s11306-016-1015-8.

Wen, B., Mei, Z., Zeng, C., Liu, S., 2017. metaX: a flexible and comprehensive software for processing metabolomics data. BMC Bioinf. 18, 183. https://doi.org/10.1186/s12859-017-1579-y.

Whiley, L., Sen, A., Heaton, J., Proitsi, P., García-Gómez, D., Leung, R., Smith, N., Thambisetty, M., Kloszewska, I., Mecocci, P., Soininen, H., Tsolaki, M., Vellas, B., Lovestone, S., Legido-Quigley, C., AddNeuroMed Consortium, 2014. Evidence of altered phosphatidylcholine metabolism in Alzheimer's disease. Neurobiol. Aging 35, 271–278. https://doi.org/10.1016/j.neurobiolaging.2013.08.001.

Wilcken, B., Wiley, V., Hammond, J., Carpenter, K., 2003. Screening newborns for inborn errors of metabolism by tandem mass spectrometry. N. Engl. J. Med. 348, 2304–2312. https://doi.org/10.1056/NEJMoa025225.

Wilkes, S.R., Beard, C.M., Kurland, L.T., Robertson, D.M., O'Fallon, W.M., 1982. The incidence of retinal detachment in Rochester, Minnesota, 1970-1978. Am. J. Ophthalmol. 94, 670–673.

Wilm, M., 2011. Principles of electrospray ionization. Mol. Cell. Proteomics 10, 009407. M111. https://doi.org/10.1074/mcp.M111.009407.

Winnike, J.H., Li, Z., Wright, F.A., Macdonald, J.M., O'Connell, T.M., Watkins, P.B., 2010. Use of pharmaco-metabonomics for early prediction of acetaminophen-induced hepatotoxicity in humans. Clin. Pharmacol. Ther. 88, 45–51. https://doi.org/10.1038/clpt.2009.240.

Wishart, D.S., 2016. Emerging applications of metabolomics in drug discovery and precision medicine. Nat. Rev. Drug Discov. 15, 473–484. https://doi.org/10.1038/nrd.2016.32.

Wojakowska, A., Marczak, Ł., Jelonek, K., Polanski, K., Widlak, P., Pietrowska, M., 2015. An optimized method of metabolite extraction from formalin-fixed paraffin-embedded tissue for GC/MS analysis. PloS One 10, e0136902. https://doi.org/10.1371/journal.pone.0136902.

Wong, W.L., Su, X., Li, X., Cheung, C.M.G., Klein, R., Cheng, C.-Y., Wong, T.Y., 2014. Global prevalence of age-related macular degeneration and disease burden projection for 2020 and 2040: a systematic review and meta-analysis. Lancet. Glob. Heal. 2, e106–e116. https://doi.org/10.1016/S2214-109X(13)70145-1.

Wong-Riley, M., 2010. Energy metabolism of the visual system. Eye Brain 2, 99. https://doi.org/10.2147/EB.S9078.

Xia, J., Wishart, D.S., 2010. MSEA: a web-based tool to identify biologically meaningful patterns in quantitative metabolomic data. Nucleic Acids Res. 38, W71–W77. https://doi.org/10.1093/nar/gkq329.

Xia, J., Broadhurst, D.I., Wilson, M., Wishart, D.S., 2013. Translational biomarker discovery in clinical metabolomics: an introductory tutorial. Metabolomics 9, 280–299. https://doi.org/10.1007/s11306-012-0482-9.

Xia, J., Sinelnikov, I.V., Han, B., Wishart, D.S., 2015. MetaboAnalyst 3.0?making metabolomics more meaningful. Nucleic Acids Res. 43, W251–W257. https://doi.org/10.1093/nar/gkv380.

Yin, P., Peter, A., Franken, H., Zhao, X., Neukamm, S.S., Rosenbaum, L., Lucio, M., Zell, A., Häring, H.-U., Xu, G., Lehmann, R., 2013. Preanalytical aspects and sample quality assessment in metabolomics studies of human blood. Clin. Chem. 59, 833–845. https://doi.org/10.1373/clinchem.2012.199257.

Yonekawa, Y., Miller, J.W., Kim, I.K., 2015. Age-related macular degeneration: advances in management and diagnosis. J. Clin. Med. 4, 343–359.

Young, S.P., Wallace, G.R., 2009. Metabolomic analysis of human disease and its

application to the eye. J. Ocul. Biol. Dis. Infor. 2, 235–242.
Young, S.P., Nessim, M., Falciani, F., Trevino, V., Banerjee, S.P., Scott, R.A.H., Murray, P.I., Wallace, G.R., 2009. Metabolomic analysis of human vitreous humor differentiates ocular inflammatory disease. Mol. Vis. 15, 1210–1217.
Yu, Z., Kastenmüller, G., He, Y., Belcredi, P., Möller, G., Prehn, C., Mendes, J., Wahl, S., Roemisch-Margl, W., Ceglarek, U., Polonikov, A., Dahmen, N., Prokisch, H., Xie, L., Li, Y., Wichmann, H.-E., Peters, A., Kronenberg, F., Suhre, K., Adamski, J., Illig, T., Wang-Sattler, R., 2011. Differences between human plasma and serum metabolite profiles. PloS One 6, e21230. https://doi.org/10.1371/journal.pone.0021230.
Yu, M., Wu, Z., Zhang, Z., Huang, X., Zhang, Q., 2015. Metabolomic analysis of human vitreous in rhegmatogenous retinal detachment associated with choroidal detachment. Investig. Opthalmology Vis. Sci. 56, 5706. https://doi.org/10.1167/iovs.14-16338.
Zemski Berry, K.A., Gordon, W.C., Murphy, R.C., Bazan, N.G., 2014. Spatial organization of lipids in the human retina and optic nerve by MALDI imaging mass spectrometry. J. Lipid Res. 55, 504–515. https://doi.org/10.1194/jlr.M044990.
Zeng, K., Xu, H., Chen, K., Zhu, J., Zhou, Y., Zhang, Q., Mantian, M., 2010. Effects of taurine on glutamate uptake and degradation in Müller cells under diabetic conditions via antioxidant mechanism. Mol. Cell. Neurosci. 45, 192–199. https://doi.org/10.1016/j.mcn.2010.06.010.
Zhang, C., Hong, H., Mendrick, D.L., Tang, Y., Cheng, F., 2015. Biomarker-based drug safety assessment in the age of systems pharmacology: from foundational to regulatory science. Biomark. Med. 9, 1241–1252. https://doi.org/10.2217/bmm.15.81.
Zhou, L., Beuerman, R.W., 2012. Tear analysis in ocular surface diseases. Prog. Retin. Eye Res. 31, 527–550. https://doi.org/10.1016/j.preteyeres.2012.06.002.
Zhou, L., Beuerman, R.W., Foo, Y., Liu, S., Ang, L.P.K., Tan, D.T.H., 2006. Characterisation of human tear proteins using high-resolution mass spectrometry. Ann. Acad. Med. Singapore 35, 400–407.
Zhou, Z., Tu, J., Zhu, Z.-J., 2017. Advancing the large-scale CCS database for metabolomics and lipidomics at the machine-learning era. Curr. Opin. Chem. Biol. 42, 34–41. https://doi.org/10.1016/j.cbpa.2017.10.033.
Ziegelstein, R., 2017. Personomics: the missing link in the evolution from precision medicine to personalized medicine. J. Personalized Med. 7, 11. https://doi.org/10.3390/jpm7040011.
Zilg, B., Bernard, S., Alkass, K., Berg, S., Druid, H., 2015. A new model for the estimation of time of death from vitreous potassium levels corrected for age and temperature. Forensic Sci. Int. 254, 158–166. https://doi.org/10.1016/j.forsciint.2015.07.020.